University of Alberta

The relationship between AMPK and PARP in inflammation

by

John W Walker ©

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ABSTRACT

A hallmark of inflammatory bowel disease (IBD) is the generation of excessive oxygen and nitrogen radicals, resulting in a spectrum of cellular damage. A principle form of cellular damage resulting from oxidant attack is DNA damage. A number of cellular mechanisms are in place to respond to DNA damage, including activation of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP). Poly(ADP-ribosyl)ation (PAR) acts as a recruitment signal to DNA repair proteins, but with chronic activation of PARP, seen in oxidatively stressed tissues, the PAR reaction depletes the cell of nicotinamide adenine dinucleotide (NAD^+), a co-factor in the synthesis of ATP. PARP inhibition is efficacious in a number of disorders where oxidative stress is involved, including IBD. However, compromising DNA repair in the course of reducing inflammation seems a costly exchange. Therefore, this thesis aims to better delineate the events downstream of PARP activation and between oxidative stress and epithelial dysfunction. Specifically, the nature of the relationship between PARP activity and the AMP-activated protein kinase (AMPK) is explored. AMPK is poised to respond to alterations in the ATP:AMP ratio by coordinating inhibition of select ATP-dependent processes while promoting processes that result in energy gain. Herein a requirement for PARP activity in the activation of AMPK by oxidative damage is demonstrated, while a pathological increase in AMPK activity in experimental colitis is described. Experiments illustrate the role of AMPK in modulating epithelial function, specifically the role of AMPK in inhibiting ion secretion, and promoting glucose uptake. Finally, the regulation of PARP by

AMPK is investigated. AMPK was found to act as a positive regulator of PARP, suggesting that the functions of AMPK may be greater than those of a simple cell rescuer. Findings indicate that PARP activity is required for AMPK activation, and that once activated, AMPK modulates epithelial function within the context of intestinal inflammation. Furthermore, PARP activity is increased by AMPK, a finding correlated with cellular apoptosis in response to sustained AMPK activation. The PARP-AMPK axis may be in place to rescue the cell from acute ATP depletion while inducing cellular apoptosis where ATP depletion, and subsequently AMPK activation, is chronic.

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LIST OF ABBREVIATIONS

3-AB	3- aminobenzamide
ACC	acetyl co-A carboxylase
Ad.CA-AMPK	constitutively active AMPK adenovirus
Ad.GFP	GFP adenovirus
AICAR	5-Aminoimidazole-4-carboxamide-1-b-D-ribofuranoside
AMPK	5' AMP-activated protein kinase
AMPKK	AMPK kinase
APC	antigen presenting cell
BBM	brush border membrane
BBMV	brush border membrane vesicles
bioNAD	biotinylated NAD^+
CD	Crohn's disease
cELISA	cell-based ELISA
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CGD	chronic granulomatous disease
ELISA	enzyme-linked immunosorbent assay
G	conductance
GBD	glycogen-binding domain
GLUT	glucose uptake transporter
IBD	inflammatory bowel disease
IBMX	3-isobutyl-1-methylxanthine
IFN-γ	interferon gamma
IL	interleukin
Isc	short-circuit current
LP	lamina propria
MALT	mucosa-associated lymphoid tissue
MAPK	mitogen-activated protein kinase
mTOR	mammalian target of rapamycin
NAD+	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor κB
PADPR	poly(ADP ribose)
PAR	poly(ADP ribose)
PARG	poly(ADP ribose) glycohydrolase
PCR	polymerase chain reaction
PD	potential difference
PJS	Peutz-Jeghers syndrome
PKA	protein kinase A
PMA	phorbol myristate acetate
RNM	reactive nitrogen metabolites
ROI	reactive oxygen intermediates

SGLT1	sodium glucose co-transporter
TLR	toll-like receptor
TNF	tumor necrosis factor
UC	ulcerative colitis

INTRODUCTION

1.1. Inflammatory Bowel Disease

Pathogenesis & Etiology. Inflammatory bowel disease (IBD) is a unifying term that describes a variety of disorders that have in common a pathological inflammatory response which is deleterious to the host. In point of fact, the aberrant inflammation is not restricted to the bowel, and may be manifested anywhere from mouth to anus, as well as in extra-intestinal locations. The term IBD is used most often to refer to the two most prevalent diseases, Crohn's disease (CD) and ulcerative colitis (UC). CD was first documented in the United States by Burrill B. Crohn, Leon Ginzburg and Gordon Oppenheimer, a team of doctors in New York City who noticed that 14 patients shared the same characteristics of a disease pattern affecting the last part of the small intestine known as the terminal ileum. Their paper on the topic was published in the Journal of the American Medical Association in 1932[1]. Since that time, the study of IBD has shown marked progress, particularly over the last two decades. This can be attributed to a number of factors, chief among them a dramatic increase in the prevalence of the disease[2], the development of exciting and powerful cellular and molecular biology techniques, and the introduction of invaluable animal models of disease to the field of study[3]. The characteristic clinical markers of the disease in the gastrointestinal tract, while variably manifested, are also fairly clear. CD can affect any site along the entire gastrointestinal tract: the lips, the oral cavity, the esophagus, the stomach, the duodenum, the jejunum, the ileum, the ileocecal valve, the cecum, the colon, the rectum and the anus. By virtue of this, the disease may go by other names, which are intended to indicate exactly where the diseased portion of the intestines is located. For example, it is commonly located in the ileum where it is referred to as ileitis or Crohn's ileitis; if it involves the ileum and the colon, it is ileocolitis. When it is in the stomach or first few loops of the intestine, it is known as gastroduodenal Crohn's disease. If it is in the next few loops of intestine, it is called jejunoileitis. If it resides in the colon, it is known as Crohn's colitis. Another term for the disease is granulomatous ileitis or enteritis, which speaks well to the immunopathology of the disease, discussed at length in a subsequent section.

Our current understanding of UC is that it strikes the large intestine or colon, unlike CD which may affect the entire GI tract, from mouth to anus. Found from the cecum to the rectum, UC tends to spread in areas that are close to where it begins, not skipping around as CD does at times. It also appears to affect primarily the mucosa and doesn't burrow through the bowel wall, another distinguishing feature from CD. As with CD, UC may be named for the areas that it strikes, going by other names such as ulcerative proctitis (affecting the rectum only) and proctosigmoiditis (the rectum and the sigmoid colon). When it takes over the entire colon, it is called panulcerative colitis or total colitis. The disease manifests itself in the mucosa. Changes at that level lead to the inflammation and ulceration that in turn cause a disturbance in the absorption of salt and water. Water malabsorption leads to diarrhea and damage to the mucosa can also lead to excessive amounts of mucous in the fecal matter. The ulcerations cause bleeding, which can lead to anemia. Abdominal pain, fever, fatigue, loss of appetite and weight loss often accompany the disease as well.

While the clinical presentation of IBD may be clear, the etiology of the disease is less so. Certainly the disease is recognized as a multifactorial disorder, including both genetic and environmental components. The pattern, or lack thereof, of heritability would strongly suggest that IBD is a polygenic disorder, and a significant body of work has and continues to be created in an attempt to elucidate the nature of the genetic variants predisposing to disease.

The IBD genes. A number of candidate genes have been implicated in the predisposition to- and development of IBD. Linkage analysis has permitted definitive disease-gene associations in at least two regions, namely for the NOD2/CARD15 gene[4] on chromosome 16 (IBD1) and the OCTN1/SLC22A4-OCT/SLC22A5 genes[5] on chromosome 5q (IBD5), both conferring increased risk for developing CD. Recently, significant associations have been reported for additional genes, including DLG5, MDR1, and TLR4 as well[6]. Of all the susceptibility genes, the NOD2/CARD15 gene mutations have been the most extensively investigated, to the extent that they are now associated with ileal disease location and a modestly earlier age of onset compared with NOD2/CARD15 wild-type CD patients.

Clinically and scientifically speaking, the identification of disease-associated genes represents a primary keystone in the elucidation of etiology and pathogenesis. With the recent successes in identifying the candidate associated genes previously mentioned, the focus of investigation now turns to deciphering the cellular and physiological ramifications of the mutations/polymorphisms. The picture emerging from the literature is not yet clear, and in some instances contradictory reports have been cited. The best studied IBD gene, the NOD2 gene has been linked with the activity of the nuclear factor kappa-B transcription factor (NF-κB), a key signaling pathway in inflammation, and one intimately linked to IBD[7]. NOD2 is in fact a protein involved in the innate immune detection of bacterial products. More specifically, NOD2 recognizes a fragment of peptidoglycan, called muramyl dipeptide that is found in the cell walls of both Gram-negative and Gram-positive bacteria. This recognition event triggers a pro-inflammatory signaling cascade regulated by the transcription factor NF-κB. NOD2 activates NF-κB in epithelial cells and macrophages, whereas NOD2 mutant 3020insC, which is associated with CD, shows an impaired ability to activate NF-κB[8]. For a time, this finding seemed paradoxical. Successful treatment strategies, including corticosteroid use[9] and the use of the more recently developed biological therapies (such as infliximab) for IBD have been focused on targeting the NF-κB pathway (although a distinction between the two therapies must be drawn here, as corticosteroids have been demonstrated to inhibit the signaling pathway, while infliximab acts downstream of any transcriptional event). Thus, the early reports that the most prevalent NOD2/CARD15 mutation conferred impairment in the NF-κB signaling cascade were confounding. In fact, a report made earlier this year would indicate the converse to be the case. Maeda et al. report that the NOD2 mutation in CD potentiates NF-κB activity[10]. This finding is more in keeping with the known pathology of IBD, but the issue remains incompletely resolved.

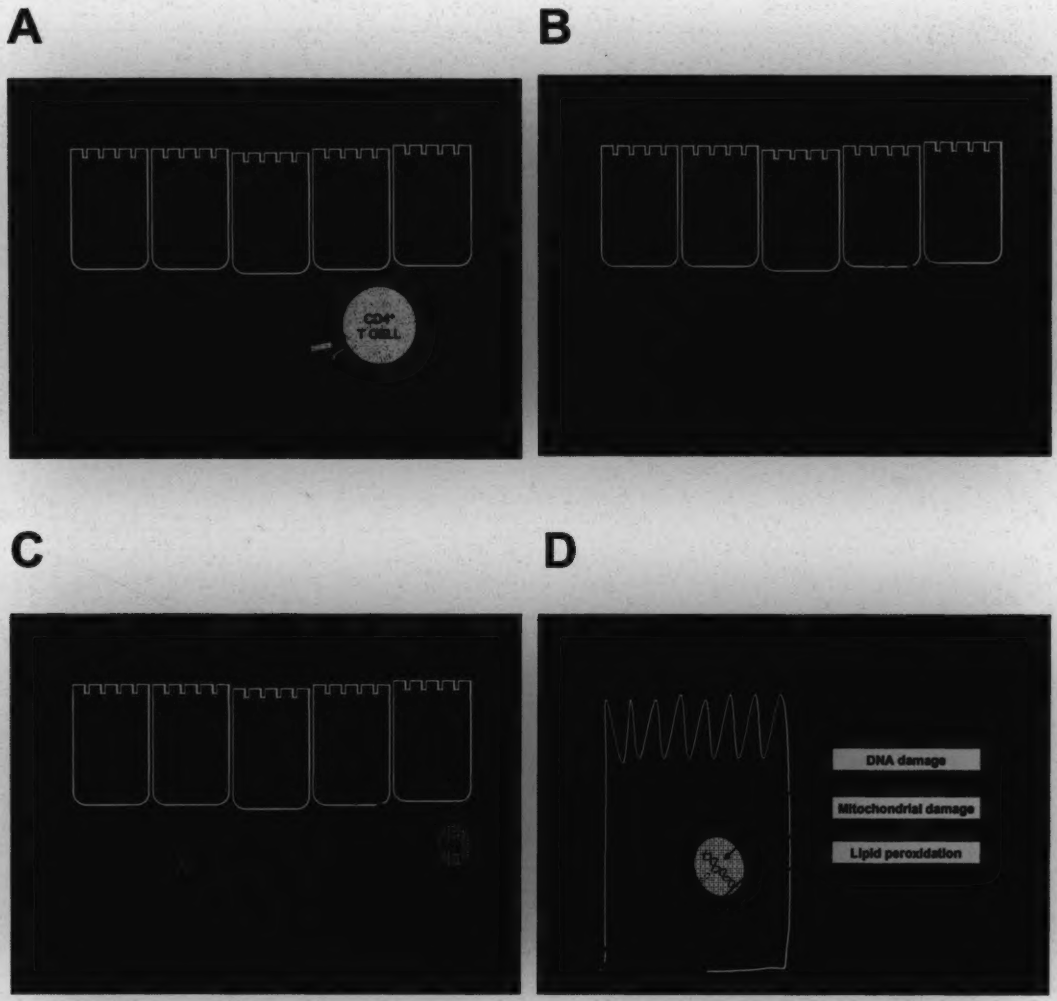
Environmental contributions to disease development. IBD is a multifactorial disease, the development of which depends upon both genetic and environmental components. Together with the genetic components previously discussed it is clear that an environmental "trigger" is required for the development of disease. The association of CD with westernization has implicated lifestyle factors in pathogenesis. A number of plausible candidate triggers have been put forward under this hypothesis, including diet, cigarette smoke and a reduced exposure to environmental antigens during childhood (reviewed by Ekbom[11]). Undoubtedly the best studied predisposing factor to disease is the microbial flora of the intestine. Evidence for a role for bacteria in the development of disease has come from a number of fronts. Antibiotics have long been an important part of the armamentarium of treatments employed in the treatment of IBD (reviewed by Guslandi[12]), and probiotics and prebiotics, both engineered to either directly or indirectly alter the microbial flora have demonstrated efficacy as well[13,14]. But perhaps the most sophisticated work addressing the role of bacteria in IBD has come from the use of mono-associated animal models of disease. In these studies, gnotobiotic animals, either genetically engineered or naturally predisposed to the development of colitis have been inoculated with single strains of bacteria. In some instances these studies have demonstrated a correlate between a bacteria and the development of disease[15]; while others have demonstrated a failed corollary[16], leading to the intriguing possibility that some, but not all, bacteria may factor in the development of IBD.

The immunopathology of IBD. The gut immune system has the challenge of responding to pathogens while remaining relatively unresponsive to food antigens and the commensal microflora. A perturbation of this balance is presumed to lead to the initiation of disease. The primary cellular layer of the gut that lies between the exterior environment and the host immune system is the epithelium, a barrier of a single-cell thickness that maintains a balance between exclusion and absorption. Neighbouring cells are joined together with tight-junctional complexes intended to provide a discrete barrier to the luminal contents. Nevertheless, those luminal constituents do reach the underlying tissues, as evidenced by the detection of macromolecular food proteins in plasma, as well as the translocation of bacteria from lumen to sub-epithelium. The intestinal epithelium can be thought of as a draped covering of the underlying lymphoid tissue (mucosal associated lymphoid tissue, or MALT) as well as the musculature and the interstitial vasculature that will speed absorbed nutrients around the body. That being said, the epithelium is far from a static barrier to the luminal constituents with which it comes into contact. The interaction between epithelium and gut content has recently been demonstrated to be one of specific immunity as well as innate defense. The intestinal epithelial cell possesses a number of receptors that enable it to sense its luminal environment. Termed toll-like receptors (TLRs) these pattern recognition receptors impart an ability to the epithelial cell to detect and respond to such discrete antigenic determinants as bacterial peptidoglycans, bacterial DNA, and bacterial flagellin (reviewed by Cario[17]). The NOD proteins discussed earlier are thought to perform the same function, but are cytosolically localized[18]. Engagement of these pattern recognition receptors leads to activation of the NF-κB pathway, and this can be viewed as the front line of mucosal defense. Should the epithelial barrier be breached by a bacterial cell, or perhaps immunogenic bacterial components, an immune response is generated in the lamina propria (LP), intended to resolve or clear the foreign antigen. The LP contains a high number and wide variety of immune cells, including IgA secreting plasma cells, dendritic cells, mast cells, eosinophils, antigen-presenting macrophage cells, $CD4^+$ T lymphocytes and an abundant population of intraepithelial $CD8^+$ lymphocytes. In a healthy individual a challenge to the host MALT would be met by an effective and limited response; in fact, sampling and clearance of luminal antigens is a constant, ongoing process. The difference between the responses in a healthy individual versus one in a person with IBD seems to be one of limit. Thus, a fundamental question remains to be answered: is pathological inflammation in IBD a case of "inflammation without infection," or is it an appropriate response to luminal antigenic stimulation that, for unknown reasons, lacks the appropriate counterbalance to terminate the immune response? This remains a critical issue to be resolved with future study. However, without knowing the precise etiology of the aberrant inflammation, a tremendous body of work has been compiled characterizing the inflammatory process particular to IBD. It is known that excess immune activation leads to an influx of inflammatory cells from the blood and to increased concentrations of cytokines, free radicals, and lipid mediators[19]. The downstream effector pathways that drive tissue injury are similar to those in immune-mediated diseases in other organs, one example being rheumatoid arthritis. For example, monoclonal antibodies to TNFα (infliximab) have been used with very good success in the treatment of both disorders[20]. A unifying hypothesis that has been put forth to explain numerous disorders of immune regulation is the concept of an imbalanced immune response to normally innocuous antigens[21]. The immune stimulation provided by the T helper (T_h) subset of lymphocytes can be broadly classified into two components, a T_h1 response and a T_h2 response. The T_h1 response produces a cytokine profile that supports inflammation and activated mainly certain T cells and macrophages, whereas the T_h2 response activates mainly B cells and immune responses that depend upon antibodies[22]. Activation of one subset of T_h lymphocytes has a counter-stimulatory or inhibitory effect on the counterpart of T_h lymphocytes. Regulation of the T_h subsets occurs at the level of cytokines; for instance, production of the canonical T_h1 cytokine IL-12 by a macrophage cell will stimulate the production of IFN-γ, which will suppress the proliferation of the T_h2 lymphocytes. Conversely, the release of the T_h2 cytokine IL-4 by a macrophage will promote the proliferation of the T_h2 subset of lymphocytes while inhibiting the production of IFN-γ, thereby suppressing the T_h1 immune response. In the absence of optimal levels of immunoregulation, an individual may develop a T_h1 or a T_h2 mediated inflammatory disorder, depending on his/her own particular T_h1/T_h2 bias, immunological history, and genetic background. CD is a T_h1 mediated inflammatory disease, while UC is a T_h2 mediated disorder. Although the cause of immune dysregulation in IBD remains unknown, a number of excellent studies have furthered our understanding of the mechanisms by which aberrant activation of the immune system leads to tissue damage. The animal models of inflammatory bowel disease provide a framework to define the immunopathogenesis of intestinal inflammation[3]. Studies in these models demonstrate the involvement of enteric microflora in the initiation and perpetuation of chronic intestinal inflammation[23]. A major pathway involves development of acquired immune responses by the interactions of $CD4^+$ T cells with antigen presenting cells (APCs). The initiating antigen remains unknown, and may in fact be a constituent of the normal bacterial flora resident in the gut. Once a pro-inflammatory cascade is initiated, a population of anti-inflammatory or suppressor T cells is recruited to the site of inflammation. In a normal or healthy individual this process would be self-resolving, with no lasting damage to the intestinal epithelium. In the case of IBD, however, the population of suppressor T cells is depleted without resolving the inflammatory response[24]. The unchecked pro-inflammatory response leads to the excessive production of pro-inflammatory cytokines and the recruitment of cytolytic cells to the inflamed site. This cascade results in micro- and macroscopic injury to the intestinal epithelium (Figure 1.1).

The role of oxidative stress in the pathophysiology of IBD. The increase in immune cell recruitment seen with IBD is accompanied by an increase in the generation of reactive oxygen intermediates (ROIs). A hallmark of the disease associated with this type of inflammation is the excessive recruitment of phagocytic leukocytes and the production of reactive metabolites (oxygen and nitrogen derived) by these cells[25,26]. The role of oxidative stress in IBD is further underscored by studies where genetic ablation of the key oxidant-detoxifying enzymes renders mice more susceptible to disease[27], a finding also in concert with evidence that sulphasalazine, a mainstay in the treatment of IBD, exerts potent antioxidant effects[28]. Our own evidence indicates that colonic inflammation in the IL-10 deficient mouse model of disease is associated with high levels of nitrosative stress[29]. When these studies are taken as a whole, the

Figure 1.1. The immunopathology of IBD

A major pathway involves development of acquired immune responses by the interactions of CD4$^+$ T cells with antigen presenting cells (A). The cognate interaction between antigen and receptor results in lymphocyte activation and the subsequent production of pro-inflammatory cytokines and chemokines (B). The elevation in chemokine concentrations attracts additional immune cells, including macrophages and suppressor lymphocytes (C). In a normal or healthy individual this process would be self-resolving. In the case of IBD the population of suppressor T cells is depleted without a resolution of the inflammation. This cascade results in micro and macroscopic injury to the intestinal epithelium (D). APC, antigen-presenting cell; IFN-γ, gamma interferon; MΦ, macrophage; TNF, tumor necrosis factor.



picture that emerges implies a critical, deleterious role for oxidative stress in the development and perpetuation of IBD.

ROI biochemistry. The primary ROI is superoxide anion ($O_2^{-}\cdot$). Formed from the single-electron reduction of molecular oxygen, $O_2^{-}\cdot$ is an oxygen free radical because it contains an unpaired electron. The generation of $O_2^{-}\cdot$ occurs through a variety of sources in both physiological and pathophysiological conditions. Within the context of IBD its generation is seen by both neutrophils and macrophages during generation of the respiratory burst. The respiratory burst, a process which involves a sudden stimulus (bacterial)-induced activation of the membrane-bound enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, evokes the release of large amounts of ROI[30]. $O_2^{-}\cdot$ generation is essential for an effective host defence against bacterial infection; to wit, dysfunction of NADPH oxidase results in chronic granulomatous disease (CGD), a syndrome characterized by severe bacterial and fungal infections. Phagocytes of the patient with CGD are unable to kill ingested microorganisms which lead to the formation of granulomas and abscesses[31]. However, the continuous overproduction of $O_2^{-}\cdot$ during inflammatory processes may also cause extensive tissue destruction.

$O_2^{-}\cdot$ is considered an unstable ROI; at physiological pH it spontaneously dismutates to H_2O_2. Thus, any system producing $O_2^{-}\cdot$ will also produce H_2O_2 and, consequently, inflammatory phagocytes also generate and release remarkable amounts of H_2O_2. H_2O_2 has been shown to directly exert non-specific damage to epithelial cells[32]. H_2O_2, however, is considered a relatively weak ROI. A series of enzymes further convert H_2O_2 to metabolites with increasing potency: hypochlorous acid (HOCl) is formed by the myeloperoxidase reaction and the hydroxyl radical ($OH\cdot$) is formed from both H_2O_2 and HOCl (discussed at length by Kruidenier et al.[33]).

Analogous to the ROIs, the reactive nitrogen metabolites, or RNMs, are derived from nitrogen; these include nitric oxide (NO) and peroxynitrite ($ONOO^{-}$). NO might have a dichotomous role as both a beneficial and detrimental molecule in intestinal inflammation[34]. However, with the spontaneous reaction of NO and $O_2^{-}\cdot$ the more toxic metabolite $ONOO^{-}$ is formed. Peroxynitrite oxidizes mitochondrial membrane lipids[35] and is also believed to damage sodium channels in the colon[36].

Cellular damage during oxidative stress. Several cellular components are affected by oxidative stress, including membrane lipids, cellular proteins and DNA. Membrane lipids are most sensitive to the ROI OH·, and in a process known as lipid peroxidation lipid hydroperoxides and aldehydes are generated[37]. Lipid peroxidation occurs in a chain reaction such that a single oxidative attack can profoundly alter the lipid membrane. These alterations come primarily in the form of altered membrane fluidity, such that cellular permeability and function is impaired. For example, changes in membrane selectivity and permeability will alter cellular metabolism[38] and cell volume homeostasis, respectively. Also of importance, the lipid aldehydes generated under oxidative attack have neutrophil chemotactic properties, thus further exacerbating intestinal inflammation[39].

Proteins are important targets of oxidative stress by virtue of their function(s) as well as the fact that they are the most abundant of the cellular constituents. Oxidants may confer alterations or ablations to protein function, as well as altered rates of turnover/degradation[40].

Finally, DNA, both nuclear and mitochondrial is damaged during attack by reactive metabolites[41]. The most common DNA modification in this setting is strand breakage, which serves to initiate the cellular DNA repair machinery. An important consequence of oxidant-mediated DNA damage (and subsequent repair,) is the depletion of cellular ATP, a process that will be discussed at length in a later section.

IBD is characterized by an extensive inflammatory infiltrate in the LP, consisting of polymorphonuclear neutrophils, eosinophils and plasma cells. However, the final steps leading from such an excessive and prolonged mucosal immune activation to tissue injury are still not well understood. Only a limited number of effector mechanisms, including oxidative stress, could account for the excessive cellular/tissue damage and chronic inflammation that is observed in IBD.

Epithelial dysfunction in IBD. Proper functioning of the intestinal epithelium is absolutely essential for health; even modest defects in its function may lead to such pathological states as diarrhea, constipation, malnutrition or dehydration, to name a few. The sequellae of IBD includes epithelial dysfunction, a prominent facet of the disease. The intestinal epithelium provides a barrier between the external environment and the host[42], and indeed, epithelial barrier function is impaired in IBD[43]. However, barrier function is but one provision of a healthy intestinal epithelium; as previously discussed the intestinal epithelium does not simply exclude, but rather must screen the contents of the gut lumen. The epithelium, in particular the enterocytes which border the lumen of the gut, forms a multifunctional unit. The epithelium balances antigen exclusion with solute and water absorption; furthermore, the epithelium can both initiate and suppress an inflammatory response. Contributions to barrier function, and a role in the inflammatory response, are important considerations in a discussion of epithelial dysfunction and IBD. In chapter four a series of experiments are presented that describes the inhibition of chloride secretion through the anion channel cystic fibrosis transmembrane conductance regulator (CFTR) in an animal model of IBD; along a similar vein, chapter five gives a picture of the possible alterations in nutrient transport, specifically glucose transport, which may accompany intestinal inflammation. The following sections of this introduction are included to provide an introduction to these topics.

The role of CFTR in colonic chloride secretion. CFTR was cloned in 1989[44]. CFTR is a member of the ATP-binding cassette (ABC) proteins, and is the product of a variety of gene mutations (>1300) that result in clinical cystic fibrosis (CF). CFTR is the predominant chloride channel in airways, sweat duct, and colon, where it is in charge of both cAMP and Ca^{2+}-activated chloride secretion[45]. The defect in CF involves an error in the post-translational modification of CFTR that results in the misfolding of the protein[46]. The gene product in CF is a functional anion channel, but in its misfolded conformation it is prematurely targeted for degradation, thus resulting in a reduced capacity for secretion by the epithelium. A defect in epithelial chloride secretion is manifested as a poorly hydrated, viscous mucous. This finding is pathognomonic for clinical CF, but also provides an apt descriptor for IBD. Indeed colonic function is similarly impaired with both CF and IBD; the likeness in epithelial dysfunction is illustrated by the phenotype of the CFTR knock-out model of CF, a model in which bowel obstruction is the first and foremost pathophysiological characteristic[47]. In chapter four a disruption in secretagogue-stimulated colonic chloride secretion in the IL-10 gene-deficient model of colitis is described; work prior to this demonstrates a similar deficiency in other models of experimental colitis[48-50]. A direct link between oxidative stress and secretory dysfunction is shown by Skinn et al. who show that nitric oxide inhibits cAMP-dependent CFTR trafficking in intestinal epithelial cells[51]. A number of defects in organ structure and function could conceivably present with the inadequate hydration of the intestinal mucosa. The mucosa which overlays the epithelium provides a barrier to bacterial attachment and invasion; in this sense it can be regarded as a key component of the intestinal barrier. Furthermore, the aqueous microenvironment of the intestinal mucosa facilitates the diffusion and absorption of essential nutrients. Chloride secretion by the intestinal epithelium is a critical mechanism of mucosal hydration, which in turn is an important determinant of intestinal health and function.

Glucose transport by the intestinal epithelium. Historically, glucose absorption by the intestinal epithelium has been solely attributed to the sodium/glucose co-transporter SGLT1[52]. In an oversimplification of the process, the process can be described as follows: glucose is taken from the lumen of the intestine by a stoichiometric ratio of 1:2; the uptake of both solutes is dependent on a sodium gradient that favors absorption, the maintenance of which depends on the function of the sodium/postassium ATPase (Na^+/K^+ ATPase). Once across the brush-border membrane of the enterocytes the glucose diffuses from the cell by the hexose transporter GLUT2, and thus this essential nutrient is made available to the systemic circulation. In this classical model for glucose absorption plasma glucose concentration is maintained at a steady-state concentration of approximately 5 mM. When the glucose concentration in the lumen is lower than in plasma, SGLT1 transports glucose uphill against its concentration gradient. While this mechanism for glucose absorption remains dogmatic, a number of studies in recent years have illustrated an alternate absorptive pathway to the one described. In 2000 Kellett and Helliwell[53] published studies that concluded that the principal route for glucose absorption is by GLUT2-mediated facilitated diffusion across the brush-border membrane, which is up to 3-fold greater than that by SGLT1. That work would open the door to subsequent studies which would elucidate this pathway as an important component of glucose uptake in health and disease (illustrated in Figure 1.2). The original work by Kellett described the post-prandial absorption of glucose, ie. during a point in time where glucose absorption by diffusion would be possible owing to the relatively high luminal concentration of the solute. The initial report of GLUT2 at the apical surface was in a rat model of diabetes[54], and indeed, it would seem to represent an adaptational response to both high luminal glucose and disease. In the fifth chapter of this thesis experimental evidence is presented that would indicate a role for stress-response protein kinases in the localization of GLUT2 to the absorptive surface. In the following sections of this preface an introduction will be made to the particular proteins of relevance to that chapter and this thesis in general.

1.2. PARP in health and disease

One half of the experimental work contained in this thesis examines the effect of the activation of the enzyme poly(ADP-ribosyl)ation polymerase (PARP) during oxidative stress in IBD. PARP is a nuclear protein involved in DNA repair; an 18 member superfamily of PARP enzymes synthesize poly(ADP-ribose) (PAR), a reversible post-translational protein modification implicated in the regulation of a number of biological functions. Poly(ADP-ribosyl)ation of PARP-target proteins serves as an important signal to additional proteins involved in DNA repair; in essence, it is the recruitment signal that collates and activates the DNA repair machinery. PARP recognizes and binds to broken-strand DNA, making it an

Figure 1.2. Glucose transport by the intestinal epithelium

There are two components to glucose transport by the intestinal epithelium. Glucose is absorbed by the enterocyte irrespective of the intracellular/luminal glucose gradient by the sodium-dependent transporter SGLT1. GLUT2, primarily recognized for its role in facilitating glucose diffusion at the basolateral membrane translocates to the apical membrane under conditions that would favor passive glucose uptake (i.e. in the post-prandial period).

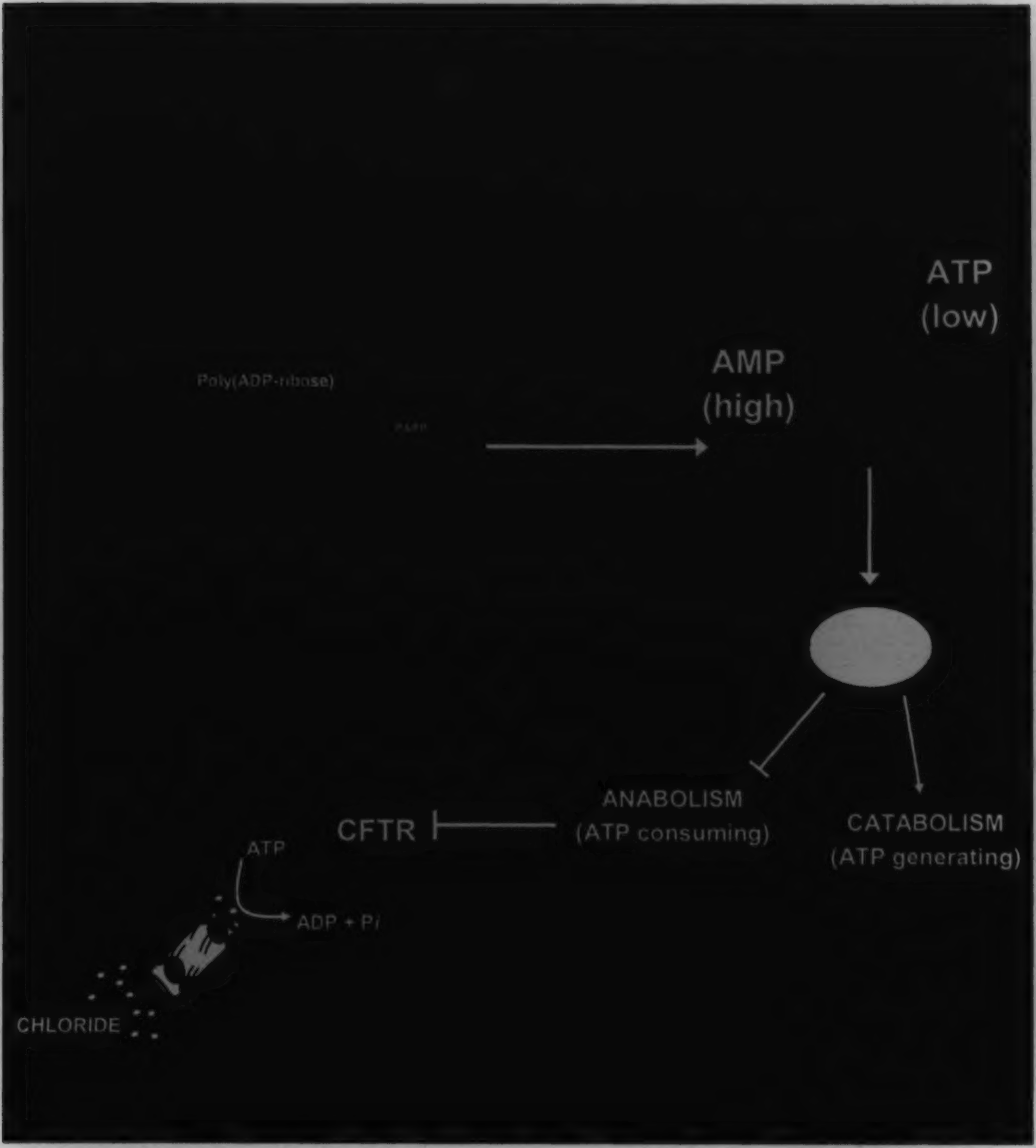
important process within the context of oxidative stress. There is no doubt that PARP activation is a crucial component to the maintenance of genomic integrity; ablative studies, specifically the PARP knockout animal models, showed enhanced sensitivity to the lethal effects of ionizing radiation and alkylating agents[55]. Furthermore, pharmacological PARP inhibitors have been shown to enhance the cytotoxicity of antitumor agents (overviewed by Tentori[56] et al.). And yet, the focus on PARP inhibition has been with an eye to therapeutic intervention, particularly in diseases where oxidative stress may be a prominent feature. The mechanism(s) of action of PARP inhibition in these various disease states is not always straightforward and perhaps not fully understood; although a common thread involves the prevention of cellular ATP depletion. That is, where a condition of chronic oxidative stress results in significant DNA damage (specifically strand breakage), there occurs an over-activation of the DNA repair proteins. Although not a pathological response in the true sense, in that the damaged DNA requires repair, it can be construed as a pathological event due to the depletion of cellular ATP that occurs during excessive PARP activation. Nicotinamide adenine dinucleotide (NAD^+) is utilized as a substrate in the poly(ADP-ribosyl)ation reaction; it is also a critical co-factor in the synthesis of ATP. Thus, a corollary can be made between the excessive activation of the enzyme PARP and subsequent ATP depletion. As stated, this theme reoccurs in a variety of pathological conditions where oxidative stress is prominent; to wit, the efficacy of inhibition of the pathway has been demonstrated in arthritis[57], cerebral ischemia[58], and diabetes[59]. Of particular importance to this thesis, PARP inhibition has also been shown to be effective in the prevention[60] and treatment[29] of IBD. PARP inhibition is effective in attenuating inflammation in experimental colitis, and this effect is likely attributable to a restoration of cellular energetics (ATP), at least in part – but why this is remains to be elucidated. Certainly a restoration of barrier function (the importance of which was touched on in an earlier section of this introduction) could be partly responsible. In fact, this has been demonstrated both *in vitro*[61] and *in vivo*[29]. However, it is most likely that restoring an adequate balance of adenylates to those cells damaged by oxidant attack has a multitude of effects. In chapter four of this thesis evidence is presented that PARP inhibition restores a defect in colonic chloride secretion through CFTR; this "makes sense," in that we know that ion secretion by this protein requires ATP. The third chapter of this thesis elaborates upon this story, by introducing a new piece to the puzzle, so to speak. The activation of the AMP-activated protein kinase (AMPK) inhibits CFTR-dependent secretion[62,63]. In fact, the study presented as chapter three describes AMPK-dependent inhibition of chloride secretion as a tenable mechanism for the hypo-secretion seen in the IL-10 gene-deficient model of colitis (depicted in Figure 1.3). Importantly, the IL-10 gene-deficient model of colitis is one of the models in which PARP inhibition has been shown to be an effective therapy. Thus, chapters two and three begin an exploration of the functional relationship between PARP and AMPK. To provide a back-drop to these studies, the following section will provide a brief introduction to AMPK.

1.3. AMPK: enzyme structure and function

AMPK is often referred to as a cellular fuel gauge, or a sensor of metabolic readiness. Under conditions of cellular stress, for example oxidative stress, the protein acts as either a rescuer to the cell or as a determinant of cellular fate. That is to say, the principle function behind AMPK activation seems to be to restore the adenylate balance to the cell, whereas the kinase would also appear to be poised to act as a molecular switch to determine cellular survival or death. AMPK is activated by any stress that depletes cellular ATP; in addition to oxidative stress, these would include metabolic poisoning, hypoxia, or nutrient deprivation. These can be regarded as pathological stresses that interfere with ATP production. A physiological stress that activates AMPK by increasing ATP consumption is exercise in skeletal muscle. AMPK coordinates the inhibition of select ATP-dependent processes while promoting, or activating, processes that result in a net energy gain. For example, ATP-consuming anabolic pathways, such as fatty acid synthesis and protein synthesis are switched-off, whereas ATP

Figure 1.3. Oxidant-dependent PARP activation increases AMPK activity resulting in impaired chloride secretion by the intestinal epithelium

A key component to oxidative stress is DNA damage. PARP activation in response to DNA strand breakage results in ATP depletion and a subsequent increase in AMPK activity. The anion channel CFTR is a substrate for AMPK. This cascade of events represents a possible mechanism by which oxidative stress results in the impairment of chloride secretion by the intestinal epithelium.



generating catabolic pathways, such as fatty acid oxidation and glycolysis, are switched-on (reviewed by Carling[64]). However, with persistent activation AMPK also appears to induce a p53-dependent metabolic checkpoint; it would appear as though AMPK is also an intrinsic regulator of the cell cycle, coordinating cellular proliferation with carbon source availability[65].

AMPK: Structure and function. Mammalian AMPK is a heterotrimeric protein consisting of a catalytic alpha subunit and regulatory beta and gamma subunits. Although the mammalian kinase is the only example that is well characterized at the biochemical level, genes encoding orthologues of all three subunits are found in all eukaryotic species whose genome sequences have been determined; thus, the existence of AMPK orthologues appears to be a fundamental feature of all eukaryotic cells. The alpha subunit (of which two are known, $\alpha1$ and $\alpha2$) contains a conventional kinase domain at the N-terminus, while the C-terminal region of the alpha subunit is required to form a complex with the regulatory subunits[66]. The beta subunits (again, there two have been identified, $\beta1$ and $\beta2$) contain two conserved regions originally termed the KIS and ASC domains[67]. The ASC domain is required for interaction with the alpha and gamma domains, while the KIS domain, interestingly, has been identified as a glycogen-binding domain. This makes physiological sense, as a high content of glycogen represses activation of AMPK[68], and conversely, a GBD would prove useful in the regulation of glycogen synthase, an important target for inhibition by AMPK[69] and one which resides in glycogen stores. The third AMPK subunit, the gamma subunit ($\gamma1$, $\gamma2$ and $\gamma3$) represents the regulatory AMP- and ATP-binding sites of the AMPK complex. A single gamma subunit possesses two discrete adenylate binding sites; at a single site binding of AMP or ATP is mutually exclusive. Depending on which of the nucleotides is bound, AMP or ATP, the kinase complex adopts a conformation that is either permissive or restrictive, respectively, of phosphorylative activity.

The regulation of AMPK by AMP. AMPK is activated by AMP in three distinct ways, all of which are antagonized by high concentrations of ATP. First, there is an allosteric activation of the kinase with gamma-subunit binding of AMP.

Typically, a five-fold increase in kinase activity is demonstrated by this mode of activation. Second, the above mentioned allosteric activation of AMPK by AMP also serves to make AMPK a "better" substrate for the upstream kinase(s). To date, a single AMPK kinase (AMPKK) has been identified, and it is the tumor-suppressor kinase LKB1[70]; worth mentioning, there is speculation that other kinases may also be acting as upstream activators of AMPK[71]. LKB1 activates AMPK through phosphorylation of a threonine residue in the alpha subunit of AMPK (Thr172)[72]. Phosphorylation of this AMPK residue causes a 50 to 100-fold increase in the activity of the kinase, and is therefore regarded as the more quantitatively important mode of regulation. The LKB1 – AMPK relationship is one of zero-order ultrasensitivity; the upstream kinase has a very low K_m for AMPK[73]. As with allosteric activation, AMPK activation by LKB1 is antagonized by high concentrations of ATP. Finally, AMP binding to AMPK also inhibits dephosphorylation of Thr172 by protein phosphatases[74]. This three-stage mode of activation lends AMPK an exquisite sensitivity to alterations in the AMP:ATP ratio within the cell. That is, a relatively small change in the initial input (ie. AMP) can effect a much greater relative change in the output of the system. If one thinks of the regulation of AMPK by AMP as a multi-step process, it becomes readily apparent that AMP is involved at each of three discrete steps within the cascade.

One of the principle findings of this thesis was the recognition that AMPK activity is increased in the intestinal epithelium of the IL-10 gene-deficient mouse. The increase is seen relative to strain-matched wild-type animals, and occurs with the development and progression of inflammation and disease in the IL-10 gene-deficient mouse. Ours is the first report of AMPK activity within the setting of experimental colitis, but it was not unexpected; oxidative stress and tissue damage provides one of the principal settings for the activation of AMPK[75]. It was the goal of this project to recognize and attempt to characterize the chain of events that begins with inflammation and oxidative damage and culminates with epithelial damage, dysfunction and ultimately, death. The experimental work contained herein hints at the potential impact of AMPK in IBD; however, it must be recognized that the involvement of AMPK in these processes is as a single player in a cast of many. With continued efforts a truer picture of the role of this and other mechanisms of pathology will emerge to clarify a complex disease.

1.4. REFERENCES

1. Crohn, B., Ginzburg, L, Oppenheimer, GD. Regional ileitis: A pathologic and clinical entity. *J Am Med Assoc* **99**, 1323-1328 (1932).
2. Binder, V. Epidemiology of IBD during the twentieth century: an integrated view. *Best Pract Res Clin Gastroenterol* **18**, 463-79 (2004).
3. Fedorak, R.N. Animal models of inflammatory bowel disease: clinical and therapeutic implications. in *Inflammatory bowel disease* (ed. J.B., K.) (Saunders, Philadelphia, 2000).
4. Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, S., Cezard, J.P., Belaiche, J., Almer, S., Tysk, C., O'Morain, C.A., Gassull, M., Binder, V., Finkel, Y., Cortot, A., Modigliani, R., Laurent-Puig, P., Gower-Rousseau, C., Macry, J., Colombel, J.F., Sahbatou, M. & Thomas, G. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* **411**, 599-603 (2001).
5. Rioux, J.D., Daly, M.J., Silverberg, M.S., Lindblad, K., Steinhart, H., Cohen, Z., Delmonte, T., Kocher, K., Miller, K., Guschwan, S., Kulbokas, E.J., O'Leary, S., Winchester, E., Dewar, K., Green, T., Stone, V., Chow, C., Cohen, A., Langelier, D., Lapointe, G., Gaudet, D., Faith, J., Branco, N., Bull, S.B., McLeod, R.S., Griffiths, A.M., Bitton, A., Greenberg, G.R., Lander, E.S., Siminovitch, K.A. & Hudson, T.J. Genetic variation in the 5q31 cytokine gene cluster confers susceptibility to Crohn disease. *Nat Genet* **29**, 223-8 (2001).
6. Vermeire, S. & Rutgeerts, P. Current status of genetics research in inflammatory bowel disease. *Genes Immun* (2005).
7. Jobin, C. & Sartor, R.B. NF-kappaB signaling proteins as therapeutic targets for inflammatory bowel diseases. *Inflamm Bowel Dis* **6**, 206-13 (2000).
8. Ogura, Y., Bonen, D.K., Inohara, N., Nicolae, D.L., Chen, F.F., Ramos, R., Britton, H., Moran, T., Karaliuskas, R., Duerr, R.H., Achkar, J.P., Brant, S.R., Bayless, T.M., Kirschner, B.S., Hanauer, S.B., Nunez, G. & Cho, J.H. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* **411**, 603-6 (2001).
9. Wild, G.E., Waschke, K.A., Bitton, A. & Thomson, A.B. The mechanisms of prednisone inhibition of inflammation in Crohn's disease involve changes in intestinal permeability, mucosal TNFalpha production and nuclear factor kappa B expression. *Aliment Pharmacol Ther* **18**, 309-17 (2003).
10. Maeda, S., Hsu, L.C., Liu, H., Bankston, L.A., Iimura, M., Kagnoff, M.F., Eckmann, L. & Karin, M. Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science* **307**, 734-8 (2005).
11. Ekbom, A. & Montgomery, S.M. Environmental risk factors (excluding tobacco and microorganisms): critical analysis of old and new hypotheses. *Best Pract Res Clin Gastroenterol* **18**, 497-508 (2004).
12. Guslandi, M. Antibiotics for inflammatory bowel disease: do they work? *Eur J Gastroenterol Hepatol* **17**, 145-7 (2005).

13. Fedorak, R.N. & Madsen, K.L. Probiotics and prebiotics in gastrointestinal disorders. *Curr Opin Gastroenterol* **20**, 146-55 (2004).
14. Fedorak, R.N. & Madsen, K.L. Probiotics and the management of inflammatory bowel disease. *Inflamm Bowel Dis* **10**, 286-99 (2004).
15. Kim, S.C., Tonkonogy, S.L., Albright, C.A., Tsang, J., Balish, E.J., Braun, J., Huycke, M.M. & Sartor, R.B. Variable phenotypes of enterocolitis in interleukin 10-deficient mice monoassociated with two different commensal bacteria. *Gastroenterology* **128**, 891-906 (2005).
16. Sydora, B.C., Tavernini, M.M., Doyle, J.S. & Fedorak, R.N. Association with selected bacteria does not cause enterocolitis in IL-10 gene-deficient mice despite a systemic immune response. *Dig Dis Sci* **50**, 905-13 (2005).
17. Cario, E. & Podolsky, D.K. Intestinal epithelial TOLLerance versus inTOLLerance of commensals. *Mol Immunol* **42**, 887-93 (2005).
18. Inohara, Chamaillard, McDonald, C. & Nunez, G. NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. *Annu Rev Biochem* **74**, 355-83 (2005).
19. Macdonald, T.T. & Pender, S.L. in *Kirsner's Inflammatory Bowel Disease* (eds. Sartor, R.B. & Sandborn, W.J.) 163-178 (Saunders, London, 2004).
20. Mitoma, H., Horiuchi, T., Hatta, N., Tsukamoto, H., Harashima, S., Kikuchi, Y., Otsuka, J., Okamura, S., Fujita, S. & Harada, M. Infliximab induces potent anti-inflammatory responses by outside-to-inside signals through transmembrane TNF-alpha. *Gastroenterology* **128**, 376-92 (2005).
21. Rook, G.A. & Brunet, L.R. Microbes, immunoregulation, and the gut. *Gut* **54**, 317-20 (2005).
22. Goldsby, R.A., Kindt, T.J. & Osborne, B.A. Kuby immunology. 40 (W.H. Freeman & Co., New York, 2000).
23. Sellon, R.K., Tonkonogy, S., Schultz, M., Dieleman, L.A., Grenther, W., Balish, E., Rennick, D.M. & Sartor, R.B. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun* **66**, 5224-31 (1998).
24. Niessner, M., Gerok, W. & Volk, B.A. Evidence for a regulatory "defect" for CD4 positive intestinal T cells in vitro in patients with IBD compared to normals. *Adv Exp Med Biol* **371B**, 1267-71 (1995).
25. Tuzun, A., Erdil, A., Inal, V., Aydin, A., Bagci, S., Yesilova, Z., Sayal, A., Karaeren, N. & Dagalp, K. Oxidative stress and antioxidant capacity in patients with inflammatory bowel disease. *Clin Biochem* **35**, 569-72 (2002).
26. Grisham, M.B. Oxidants and free radicals in inflammatory bowel disease. *Lancet* **344**, 859-61 (1994).
27. Krieglstein, C.F., Cerwinka, W.H., Laroux, F.S., Salter, J.W., Russell, J.M., Schuermann, G., Grisham, M.B., Ross, C.R. & Granger, D.N. Regulation of murine intestinal inflammation by reactive metabolites of oxygen and nitrogen: divergent roles of superoxide and nitric oxide. *J Exp Med* **194**, 1207-18 (2001).

28. Pearson, D.C., Jourd'heuil, D. & Meddings, J.B. The anti-oxidant properties of 5-aminosalicylic acid. *Free Radic Biol Med* **21**, 367-73 (1996).
29. Jijon, H.B., Churchill, T., Malfair, D., Wessler, A., Jewell, L.D., Parsons, H.G. & Madsen, K.L. Inhibition of poly(ADP-ribose) polymerase attenuates inflammation in a model of chronic colitis. *Am J Physiol Gastrointest Liver Physiol* **279**, G641-51 (2000).
30. Hampton, M.B., Kettle, A.J. & Winterbourn, C.C. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* **92**, 3007-17 (1998).
31. Jurkowska, M., Bernatowska, E. & Bal, J. Genetic and biochemical background of chronic granulomatous disease. *Arch Immunol Ther Exp (Warsz)* **52**, 113-20 (2004).
32. Mulier, B., Rahman, I., Watchorn, T., Donaldson, K., MacNee, W. & Jeffery, P.K. Hydrogen peroxide-induced epithelial injury: the protective role of intracellular nonprotein thiols (NPSH). *Eur Respir J* **11**, 384-91 (1998).
33. Kruidenier, L., Kuiper, I., Lamers, C.B. & Verspaget, H.W. Intestinal oxidative damage in inflammatory bowel disease: semi-quantification, localization, and association with mucosal antioxidants. *J Pathol* **201**, 28-36 (2003).
34. Kubes, P. & McCafferty, D.M. Nitric oxide and intestinal inflammation. *Am J Med* **109**, 150-8 (2000).
35. Gadelha, F.R., Thomson, L., Fagian, M.M., Costa, A.D., Radi, R. & Vercesi, A.E. Ca2+-independent permeabilization of the inner mitochondrial membrane by peroxynitrite is mediated by membrane protein thiol cross-linking and lipid peroxidation. *Arch Biochem Biophys* **345**, 243-50 (1997).
36. Bauer, M.L., Beckman, J.S., Bridges, R.J., Fuller, C.M. & Matalon, S. Peroxynitrite inhibits sodium uptake in rat colonic membrane vesicles. *Biochim Biophys Acta* **1104**, 87-94 (1992).
37. Gutteridge, J.M. Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clin Chem* **41**, 1819-28 (1995).
38. Chen, J.J., Bertrand, H. & Yu, B.P. Inhibition of adenine nucleotide translocator by lipid peroxidation products. *Free Radic Biol Med* **19**, 583-90 (1995).
39. Curzio, M., Esterbauer, H., Poli, G., Biasi, F., Cecchini, G., Di Mauro, C., Cappello, N. & Dianzani, M.U. Possible role of aldehydic lipid peroxidation products as chemoattractants. *Int J Tissue React* **9**, 295-306 (1987).
40. Dean, R.T., Fu, S., Stocker, R. & Davies, M.J. Biochemistry and pathology of radical-mediated protein oxidation. *Biochem J* **324 (Pt 1)**, 1-18 (1997).
41. Yakes, F.M. & Van Houten, B. Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. *Proc Natl Acad Sci U S A* **94**, 514-9 (1997).
42. Medzhitov, R. & Janeway, C., Jr. Innate immunity. *N Engl J Med* **343**, 338-44 (2000).

43. Bjarnason, I., MacPherson, A. & Hollander, D. Intestinal permeability: an overview. *Gastroenterology* **108**, 1566-81 (1995).
44. Kerem, B., Rommens, J.M., Buchanan, J.A., Markiewicz, D., Cox, T.K., Chakravarti, A., Buchwald, M. & Tsui, L.C. Identification of the cystic fibrosis gene: genetic analysis. *Science* **245**, 1073-80 (1989).
45. Barrett, K.E. & Keely, S.J. Chloride secretion by the intestinal epithelium: molecular basis and regulatory aspects. *Annu Rev Physiol* **62**, 535-72 (2000).
46. White, M.B., Amos, J., Hsu, J.M., Gerrard, B., Finn, P. & Dean, M. A frame-shift mutation in the cystic fibrosis gene. *Nature* **344**, 665-7 (1990).
47. Grubb, B.R. & Gabriel, S.E. Intestinal physiology and pathology in gene-targeted mouse models of cystic fibrosis. *Am J Physiol* **273**, G258-66 (1997).
48. Goldhill, J.M., Burakoff, R., Donovan, V., Rose, K. & Percy, W.H. Defective modulation of colonic secretomotor neurons in a rabbit model of colitis. *Am J Physiol* **264**, G671-7 (1993).
49. Bell, C.J., Gall, D.G. & Wallace, J.L. Disruption of colonic electrolyte transport in experimental colitis. *Am J Physiol* **268**, G622-30 (1995).
50. Asfaha, S., Bell, C.J., Wallace, J.L. & MacNaughton, W.K. Prolonged colonic epithelial hyporesponsiveness after colitis: role of inducible nitric oxide synthase. *Am J Physiol* **276**, G703-10 (1999).
51. Skinn, A.C. & MacNaughton, W.K. Nitric oxide inhibits cAMP-dependent CFTR trafficking in intestinal epithelial cells. *Am J Physiol Gastrointest Liver Physiol* **289**, G739-44 (2005).
52. Wright, E.M., Martin, M.G. & Turk, E. Intestinal absorption in health and disease--sugars. *Best Pract Res Clin Gastroenterol* **17**, 943-56 (2003).
53. Kellett, G.L. & Helliwell, P.A. The diffusive component of intestinal glucose absorption is mediated by the glucose-induced recruitment of GLUT2 to the brush-border membrane. *Biochem J* **350 Pt 1**, 155-62 (2000).
54. Corpe, C.P., Basaleh, M.M., Affleck, J., Gould, G., Jess, T.J. & Kellett, G.L. The regulation of GLUT5 and GLUT2 activity in the adaptation of intestinal brush-border fructose transport in diabetes. *Pflugers Arch* **432**, 192-201 (1996).
55. Masutani, M., Nakagama, H. & Sugimura, T. Poly(ADP-ribose) and carcinogenesis. *Genes Chromosomes Cancer* **38**, 339-48 (2003).
56. Tentori, L. & Graziani, G. Chemopotentiation by PARP inhibitors in cancer therapy. *Pharmacol Res* **52**, 25-33 (2005).
57. Szabo, C., Virag, L., Cuzzocrea, S., Scott, G.S., Hake, P., O'Connor, M.P., Zingarelli, B., Salzman, A. & Kun, E. Protection against peroxynitrite-induced fibroblast injury and arthritis development by inhibition of poly(ADP-ribose) synthase. *Proc Natl Acad Sci U S A* **95**, 3867-72 (1998).
58. Eliasson, M.J., Sampei, K., Mandir, A.S., Hurn, P.D., Traystman, R.J., Bao, J., Pieper, A., Wang, Z.Q., Dawson, T.M., Snyder, S.H. & Dawson, V.L. Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. *Nat Med* **3**, 1089-95 (1997).

59. Mabley, J.G., Suarez-Pinzon, W.L., Hasko, G., Salzman, A.L., Rabinovitch, A., Kun, E. & Szabo, C. Inhibition of poly (ADP-ribose) synthetase by gene disruption or inhibition with 5-iodo-6-amino-1,2-benzopyrone protects mice from multiple-low-dose-streptozotocin-induced diabetes. *Br J Pharmacol* **133**, 909-19 (2001).
60. Di Paola, R., Mazzon, E., Patel, N.S., Genovese, T., Muia, C., Thiemermann, C., De Sarro, A. & Cuzzocrea, S. Beneficial effects of GW274150 treatment on the development of experimental colitis induced by dinitrobenzene sulfonic acid. *Eur J Pharmacol* **507**, 281-9 (2005).
61. Kennedy, M., Denenberg, A.G., Szabo, C. & Salzman, A.L. Poly(ADP-ribose) synthetase activation mediates increased permeability induced by peroxynitrite in Caco-2BBe cells. *Gastroenterology* **114**, 510-8 (1998).
62. Hallows, K.R., Raghuram, V., Kemp, B.E., Witters, L.A. & Foskett, J.K. Inhibition of cystic fibrosis transmembrane conductance regulator by novel interaction with the metabolic sensor AMP-activated protein kinase. *J Clin Invest* **105**, 1711-21 (2000).
63. Hallows, K.R., Kobinger, G.P., Wilson, J.M., Witters, L.A. & Foskett, J.K. Physiological modulation of CFTR activity by AMP-activated protein kinase in polarized T84 cells. *Am J Physiol Cell Physiol* **284**, C1297-308 (2003).
64. Carling, D. AMP-activated protein kinase: balancing the scales. *Biochimie* **87**, 87-91 (2005).
65. Jones, R.G., Plas, D.R., Kubek, S., Buzzai, M., Mu, J., Xu, Y., Birnbaum, M.J. & Thompson, C.B. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol Cell* **18**, 283-93 (2005).
66. Crute, B.E., Seefeld, K., Gamble, J., Kemp, B.E. & Witters, L.A. Functional domains of the alpha1 catalytic subunit of the AMP-activated protein kinase. *J Biol Chem* **273**, 35347-54 (1998).
67. Jiang, R. & Carlson, M. The Snf1 protein kinase and its activating subunit, Snf4, interact with distinct domains of the Sip1/Sip2/Gal83 component in the kinase complex. *Mol Cell Biol* **17**, 2099-106 (1997).
68. Wojtaszewski, J.F., MacDonald, C., Nielsen, J.N., Hellsten, Y., Hardie, D.G., Kemp, B.E., Kiens, B. & Richter, E.A. Regulation of 5'AMP-activated protein kinase activity and substrate utilization in exercising human skeletal muscle. *Am J Physiol Endocrinol Metab* **284**, E813-22 (2003).
69. Hudson, E.R., Pan, D.A., James, J., Lucocq, J.M., Hawley, S.A., Green, K.A., Baba, O., Terashima, T. & Hardie, D.G. A novel domain in AMP-activated protein kinase causes glycogen storage bodies similar to those seen in hereditary cardiac arrhythmias. *Curr Biol* **13**, 861-6 (2003).
70. Woods, A., Johnstone, S.R., Dickerson, K., Leiper, F.C., Fryer, L.G., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M. & Carling, D. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol* **13**, 2004-8 (2003).
71. Hong, S.P., Leiper, F.C., Woods, A., Carling, D. & Carlson, M. Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. *Proc Natl Acad Sci U S A* **100**, 8839-43 (2003).

72. Hawley, S.A., Davison, M., Woods, A., Davies, S.P., Beri, R.K., Carling, D. & Hardie, D.G. Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. *J Biol Chem* **271**, 27879-87 (1996).

73. Hardie, D.G., Salt, I.P., Hawley, S.A. & Davies, S.P. AMP-activated protein kinase: an ultrasensitive system for monitoring cellular energy charge. *Biochem J* **338 (Pt 3)**, 717-22 (1999).

74. Davies, S.P., Helps, N.R., Cohen, P.T. & Hardie, D.G. 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. *FEBS Lett* **377**, 421-5 (1995).

75. Choi, S.L., Kim, S.J., Lee, K.T., Kim, J., Mu, J., Birnbaum, M.J., Soo Kim, S. & Ha, J. The regulation of AMP-activated protein kinase by H(2)O(2). *Biochem Biophys Res Commun* **287**, 92-7 (2001).

CHAPTER 2

PARP activity is required for oxidant-dependent AMPK activation in intestinal epithelial cells

2.1. INTRODUCTION

Reactive oxygen species in inflammatory bowel disease

Inflammatory bowel disease (IBD) is a complex, multifactorial disorder that includes both genetic and environmental etiological components. The two most commonly described variants of IBD are Crohn's disease (CD) and ulcerative colitis (UC). While the cause of these diseases remains unknown, there is strong evidence that would indicate an aberrant immune response to normal luminal constituents, possibly bacteria or bacterial products, to be a critical factor in the development of disease. Research into the pathogenesis of CD using animal models supports a role for T_h1-mediated immune responses[1], and inhibition of the generation of a T_h1 response is known to prevent disease[2,3]. A hallmark of the disease associated with this type of inflammation is the excessive recruitment of phagocytic leukocytes and the production of reactive metabolites (oxygen and nitrogen derived) by these cells[4,5]. The role of oxidative stress in IBD is further underscored by studies where genetic ablation of the key oxidant-detoxifying enzymes renders mice more susceptible to disease [6], a finding also in concert with evidence that sulphasalazine, a mainstay in the treatment of IBD, exerts potent antioxidant effects[7]. Our own evidence indicates that colonic inflammation in the IL-10 deficient mouse model of disease is associated with high levels of nitrosative stress[8]. When these studies are taken as a whole, the picture that emerges implies a critical, deleterious role for oxidative stress in the development and perpetuation of IBD.

Pathophysiologic role of oxidative stress-induced PARP activation

Oxygen- and nitrogen-derived reactive species react with membrane lipids, intracellular proteins and DNA resulting in changes to epithelial architecture and function within the gut. In inflicting cellular damage oxidative species act somewhat indiscriminately, but one of the principal ramifications of oxidative stress in intestinal inflammation is the generation of a cellular energy deficit. There exist multiple mechanisms by which oxidative stress could result in a depletion of intracellular energy stores, one of which is the activation of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP)[9]. This enzyme acts to transfer the ADP-ribose moiety of NAD^+ to various chromatin proteins[10]. Szabo and Dawson[10] demonstrated a process whereby chronic activation of PARP can potentially lead to a depletion of cellular NAD^+ and, because NAD^+ is a critical co-factor in ATP synthesis, a decline in cellular ATP levels. Due in part to this fact, PARP inhibition has proven to be efficacious in a number of disorders that include a component of oxidative stress, including ischemia-reperfusion injury, diabetes and rheumatoid arthritis (reviewed in Beneke et al.[11]). In 1998 Kennedy et al.[12] reported that PARP activation mediated an impairment in intestinal barrier function (the maintenance of which requires ATP) in response to the potent oxidant peroxynitrite. That paper provided the impetus for a study by our group in which it was demonstrated that the inhibition of PARP attenuated colonic inflammation in the IL-10 deficient mouse[8]. The IL-10 deficient mouse exhibits increased intestinal permeability[13], and current opinion holds that a deranged epithelial barrier may be a contributing factor to the development and perpetuation of the colonic disease seen in this model of IBD. In our study PARP inhibition normalized the permeability defect in IL-10 deficient mice and this finding was correlated with a marked improvement of colonic inflammatory disease. Thus, excessive PARP activation, as seen in animal models of IBD, has been demonstrated to impair gut barrier function, while inhibition of the enzyme ameliorates disease.

Role of AMPK in the response to cellular stress

Ultimately the inverse relationship between sustained PARP activation and intracellular ATP results in a failure of energy (ATP)-dependent cellular processes. In situations where oxidative stress is chronic (ie. the inflamed intestine) the cellular response must be balanced; the utilization of critical cellular repair processes such as PARP-dependent DNA repair must not outstrip the cell's ability to maintain sufficient stores of ATP. Therefore it is critical for the cell to sense and respond to alterations in energy status. The AMP-activated protein kinase (AMPK) appears to act as a master switch capable of maintaining energy balance within cells by regulating rates of both ATP-consuming and ATP-generating pathways[14]. Present in all eukaryotic cells, AMPK is a serine/threonine kinase that exists as a heterotrimer composed of a catalytic α subunit and regulatory β and γ subunits[15]. Activation of AMPK involves an allosteric mechanism by which AMP binds to AMPK, in addition to phosphorylation of a threonine residue on the catalytic subunit of AMPK catalysed by an upstream kinase, LKB1[16]. Recently, we characterized the role of AMPK in the intestinal epithelium as it pertained to ion secretion through the cystic fibrosis transmembrane conductance regulator (CFTR), and demonstrated that this energy-dependent ion secretion process was inhibited by AMPK[17]. In that study we demonstrated hyposecretion by the inflamed colon of the IL-10 deficient mouse and rescued the defect by treatment with a pharmacological inhibitor of AMPK. What is more, treatment of wild-type animal tissue with the AMPK activator 5-aminoimidazole-4-carboxamide riboside (AICAR) mimicked the hyposecretion evident in IL-10 deficient mice. Thus, we showed that in an animal model of IBD where oxidative stress and PARP activation had previously been shown to result in a failure of ATP-dependent processes the inhibition of AMPK could act to restore function. In our current study we set out to link the activation of PARP by oxidative stress with the subsequent activation of AMPK, and to determine if a defect in chloride secretion attributable to AMPK would be possible by the pharmacological inhibition of PARP.

2.2 EXPERIMENTAL PROCEDURES

Animals

129 Sv/Ev mice were housed behind a barrier under specific pathogen-free conditions. The mice had *ad libitum* access to autoclaved 9% fat rodent blocs and sterile filtered water. The facility's sanitation was verified by Health Sciences Lab Animal Services at the University of Alberta (Edmonton, Alberta, Canada). All experiments were performed according to the institutional guidelines for the care and use of laboratory animals in research and with the permission of the local ethics committee.

Western Blotting

Jejunal mucosa was collected by scraping with a microscope slide and suspended in 0.5 ml Mono-Q buffer. Following collection, tissue suspensions were sonicated on ice for 2 x 15 sec. Protein concentrations were determined using the Bradford method and samples diluted to equivalent concentrations. Duplicate samples were separated by SDS-PAGE and either Coomassie-stained (to ensure even loading of lanes) or transferred onto PVDF membrane (Millipore, Billerica, MA). Membranes were blocked for 2 hr with 3% skim milk-TTBS (20 mM Tris, 0.5 M NaCl, 0.05% Tween 20, pH 7.4) and incubated overnight at 4°C with primary antibody (diluted to manufacturer's specifications). Membranes were then washed 3 times with water and incubated for 2 hr with goat anti-rabbit secondary antibodies (R&D, Minneapolis, MN; 1:4000 dilution), followed by 2 washes with TTBS. Autoradiography was performed on Kodak X-OMAT AR film using a chemiluminescence kit (Lumi-light, Amersham, Piscataway, NJ). Two antibodies were employed for Western blot in this study, recognizing phospho-Acetyl Co-A Carboxylase (Cell Signaling Technologies, Beverly, MA) and poly(ADP-ribose) (Tulip biolabs, West Point, PA) respectively.

AMPK Activity Assay

AMPK enzyme activity was assayed as previously described[18]. Subsequent to experimental treatment, an equivalent volume of ice-cold 2X homogenization buffer (in mmol/L): 50 Tris HCl, 250 mannitol, 1 EDTA, 1 EGTA, 50 NaF, 5 $Na_4P_2O_7 \cdot 10\ H_2O$, 1 PMSF, 1 DTT, and 10% glycerol, 0.1% Triton X-100, and 1 μl/ml protease inhibitor cocktail (Sigma, St. Louis, MO) was added directly to the culture media prior to collecting cells by scraping and snap-freezing in liquid nitrogen. The crude homogenate was sonicated with 4 pulses of 3 sec each before centrifugation at 18000 g for 3 min. Protein concentrations were then determined using the Bradford method, and each sample was diluted accordingly to an equivalent protein concentration. To 200 μL of that sample was added 2 μL α-AMPK antibody (Cell Signaling Technologies), and the immunoprecipitation was incubated overnight at 4°C with gentle mixing. After 12 hr immunoprecipitation, 30 μL of protein A beads (50% slurry, Santa Cruz biotechnology, Santa Cruz, CA) were added to each sample and incubated for 2 hr at 4°C with gentle mixing. The assay was begun with the addition of immunoprecipitated enzyme to assay buffer (in mM: 80 HEPES buffer, 160 NaCl, 1.6 EDTA, 200 μM SAMS peptide (Alberta Peptide Institute, Edmonton, AB, Canada), 200 μM AMP, 200 μM ATP, 16% glycerol, 0.1% Triton X-100 and 0.5 μCi [γ-32^P]-ATP per sample). Following addition of enzyme to the reaction tube, samples were vortexed 5 sec and incubated for 10 min at 30°C. Following incubation, the reaction mixture was vortexed and spotted on P81 Whatman filter paper (Fisher Scientific, Pittsburgh, PA) briefly allowed to dry, and washed three times in 1% perchloric acid before a single wash in acetone. After sufficient time to allow the filter papers to air dry, they were immersed in a scintillant-fluor cocktail and the activity of each sample was measured in a Beckman scintillation counter. Unless otherwise listed, all reagents used in this assay were purchased from Sigma (St. Louis, MO).

Epithelial Chloride Secretion

Mice were sacrificed by cervical dislocation, and a segment of proximal colon was removed. Colonic tissue was mounted in lucite chambers exposing mucosal and serosal surfaces to 10 mL of oxygenated Krebs buffer (in mmol/L: 115 NaCl, 8 KCl, 1.25 $CaCl_2$, 1.2 $MgCl_2$, 2 KH_2PO_4, 25 $NaHCO_3$; pH 7.35). The buffers were maintained at 37°C by a heated water jacket and circulated by CO_2/O_2. Fructose (10 mmol/L) was added to the serosal and mucosal sides as a metabolic fuel source for the colonic tissue. The spontaneous transepithelial potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate short-circuit current (Isc) with an automatic voltage clamp (DVC 1000 World Precision Instruments, New Haven, CT), except for 5-10 sec every 5 min when PD was measured by removing the voltage clamp. Tissue ion conductance (G) was calculated from PD and Isc according to Ohm's Law.

For the measurement of basal chloride fluxes, the tissue was clamped at zero voltage by continuously introducing an appropriate Isc with an automatic voltage clamp, except for 5-10 sec every 5 min when PD was measured by removing the voltage clamp. Tissue pairs were matched for conductance and discarded if conductance varied by > 20%. 5 μCi ^{36}Cl was added to the serosal side after mounting and the tissue was allowed to equilibrate for 20 min. Unidirectional flux from serosal-to-mucosal surface was determined for paired tissues by measuring four consecutive five-min fluxes prior to the addition of forskolin (10^{-4} M) and two five-min fluxes following the addition of the secretagogue.

Chloride flux in response to forskolin is reported as the difference between the averaged values of the four consecutive five-min fluxes prior to the addition of the secretagogue and the averaged values of the two consecutive five-min fluxes post-stimulation.

Cell Culture

HT-29 cells at passages 30-42 were grown as monolayers in RPMI 1640 medium with L -glutamine, without sodium bicarbonate, supplemented with 5% newborn calf serum. For subculture, a cell suspension was obtained from confluent monolayers by exposing the monolayers to 0.25% trypsin and 0.9 mM EDTA in Ca^+ and Mg^+ free phosphate buffered saline.

Statistical Analysis

Data are expressed as means ± SE, and statistical analyses were performed using the statistical software SigmaStat (Jandel Corp, San Rafael, CA) Differences between mean values were evaluated by analysis of variance or paired *t* test where appropriate. Specific differences were tested using Student-Newman-Keuls test.

2.3 RESULTS

Pharmacological inhibition of PARP prevents the activation of AMPK by treatment with exogenous oxidants

To reproduce the environment of oxidative stress seen in IBD we treated cultured intestinal epithelial cells (HT-29) with varying concentrations of H_2O_2 (0-5 mM) for the time periods indicated. We[17] and others[19] have previously demonstrated the activation of AMPK in intestinal epithelial cells by this method, and it is furthermore recognized that H_2O_2 is a potent inducer of DNA damage – dependent PARP activation[20]. Our hypothesis held that the activation of AMPK within the context of intestinal oxidative stress would depend largely on the activation of PARP and the subsequent depletion of intracellular ATP. Therefore, inhibition of PARP within these experimental parameters should result in the inhibition or prevention of AMPK activity. To investigate our hypothesis we stimulated PARP activity in intestinal epithelial cells in the presence and absence of the PARP inhibitors PJ34 (0-10 μM, as indicated) and 3-AB (2.5 mM) and assessed AMPK activity by Western blot analysis as well as by specific kinase assay. As seen in figure 2.1, there exists a significant degree of basal PARP activity in HT-29 cells, as witnessed by the level of poly(ADP-ribosylation) even in the absence of exogenous stimulation by H_2O_2. Nonetheless, stimulation with 5 mM H_2O_2 increases the level of PARP activity to a level that appears maximal at 15 minutes, as no further increase in activity is observed beyond this time point. Within this experiment, the potency of the PARP inhibitor PJ34 is demonstrated by the complete absence of poly(ADP)-ribosylation at concentrations as low as 0.5 μM. Figure 2.2.A represents a similar experiment to that depicted in the previous figure, with the exception that the time of treatment of HT_{29} cells with H_2O_2 was fixed (30 minutes) while the oxidant concentration was varied. In this experiment we investigated the effect of PARP inhibition (PJ34, 10 μM) on AMPK activity within the context of oxidative stress. As has been previously shown, the phosphorylation state of the well characterized AMPK substrate acetyl co-A carboxylase (ACC) serves as a surrogate marker for the activity of AMPK[18,21]. By

Western blot analysis we reveal that AMPK activity increases dose-dependently with oxidant treatment, as evidenced by the increasing phosphorylation of ACC (Figure 2.2.A). Within this experiment we included a treatment group exposed to the highest level of oxidative stress, yet pre-treated with the PARP inhibitor PJ34 (10 μM, 30 minutes prior to treatment with H_2O_2). Figure 2.2.A illustrates the inhibition of oxidant-dependent ACC phosphorylation with the inhibition of PARP. Accepting this finding as an indirect indication that PARP activity was required for AMPK activity, we next attempted to reinforce our hypothesis by employing a direct kinase activity assay. Our experimental conditions in figure 2.2.B were similar to those in figures 2.1 and figure 2.2.A, with the exception that 3-AB (2 mM) was substituted for PJ34. It was felt that the inclusion of a second PARP inhibitor at this point, specifically 3-AB which is well characterized in the literature and possessing a structure distinct from that of PJ34, would strengthen our study. Again, pre-treatment of HT-29 cells with 3-AB was for 30 minutes prior to oxidant treatment (1 mM H_2O_2) and as a control we included a treatment group pre-treated with the inactive structural analogue to 3-AB, aminobenzoic acid (ABA, 2 mM). The results of the kinase assay under these conditions are unequivocal and support the previously described Western blot findings. Consistent with reports in the literature we are able to generate a nearly two-fold increase in AMPK activity by stimulation with H_2O_2. This increase in AMPK activity is prevented by pre-treating the HT-29 cells with 3-AB, while the control pre-treatment did not provide a statistically significant reduction in oxidant-dependent activity.

Pharmacological inhibition of PARP restores an AMPK-dependent defect in colonic chloride secretion in the IL-10 deficient model of colitis

In 2003 we demonstrated a relationship between AMPK activity and colonic chloride hyposecretion[17]. Our work provided a clinical correlate to the work of Hallows et al.[22,23], who were first to demonstrate the physiological and inhibitory nature of the relationship between AMPK and the cystic fibrosis transmembrane conductance regulator (CFTR). Working with the IL-10 deficient mouse, our group demonstrated that a defect in colonic cAMP-dependent chloride secretion (primarily attributable to CFTR) could be rescued by inhibition of AMPK, and conversely, that activation of the kinase by pharmacological means mimicked the colitic defect in otherwise healthy wild-type animals[17]. If the activation of AMPK by oxidative stress was dependent on the activity of PARP, as was demonstrated by our *in vitro* experiments, treatment of the IL-10 deficient mouse with a PARP inhibitor should bring about a restoration of function pertaining to chloride secretion. In fact, *in vivo* treatment of IL-10 deficient animals with the PARP inhibitor 3-AB (20 mg/kg intraperitoneal injection), as well as *ex vivo* treatment of colonic explants with the PARP inhibitor GPI-6150 (100 μM) were both effective in reversing the secretory defect (Figure 2.3). The effect of PARP inhibition on cAMP-dependent chloride secretion within the inflammatory milieu was acute, as demonstrated by the fact that a single intraperitoneal injection 1 hour prior to sacrifice was capable of restoring ion secretion. Similarly, restoration of function was evident with just 15 minutes *ex vivo* treatment of colonic explant. Treatment with the two PARP inhibitors (3-AB and GPI-6150) did not differ to a statistically significant degree with respect to efficacy, although the mean increase in chloride secretion with GPI-6150 treatment was measurably greater than with 3-AB treatment. It is possible that treatment of isolated colonic tissue presented a more accessible target for pharmacological PARP inhibition than did intraperitoneal injection, and furthermore, GPI-6150 has been shown to act with greater potency than 3-AB[24]. Nonetheless, that the two PARP inhibitors act to restore an AMPK-dependent defect in chloride secretion argues strongly for our argument that PARP inhibition constrains AMPK activation. Of note, while not illustrated in Figure 2.3, treatment of wild-type colonic explant tissue with GPI-6150, as well as intraperitoneal injection of wild-type animals with 3-AB was performed and these treatments did not alter either basal nor cAMP-dependent chloride secretion in these groups.

2.4. DISCUSSION

The greater part of this work was conducted to demonstrate a link between the activation of PARP and subsequent AMPK activity, and to that end, cAMP-dependent chloride secretion by CFTR was measured as a useful surrogate physiological marker for inhibition of epithelial function by AMPK[17]. However, the recognition that pharmacological PARP inhibition restores colonic epithelial chloride secretion is noteworthy in its own regard. Colonic chloride secretion is a critical determinant of mucosal hydration, and in its absence (as seen with the IL-10 deficient mouse) colonic function is compromised. Paradoxically, through a poorly hydrated colonic mucosa certainly water resorption by the bowel would be compromised, which might in part contribute to the diarrhea frequently observed in IBD. Furthermore, epithelial barrier function, likely a determinant in the pathogenesis of disease, also requires sufficient hydration of the mucosa. In this regard, hyposecretion by the colonic epithelium would be expected to compromise a key component of the bowel's innate immune system. And lastly, the poorly hydrated, viscous mucus that lines the epithelium in the absence of chloride secretion creates an environment conducive to bacterial colonization and overgrowth. This too may play a role in the perpetuation of colonic disease in IBD.

Activation of PARP is an important factor in the pathogenesis of various inflammatory diseases[25] and similarly, while the role of AMPK in rescuing the cell from ATP depletion is indispensable, it can also be said that chronic activation of this mechanism creates a state of cellular dysfunction[17]. The contributions of both of these proteins to the pathogenesis of inflammatory disease is further underscored by their promise as potential targets for therapeutic inhibition[25,26]. Here we link these two important proteins within the greater cascade of oxidant-dependent stress responses. Specifically, with this study we demonstrate a requirement for PARP activity in the activation of AMPK by oxidative stress.

One of the primary therapeutic mechanisms of PARP inhibition is the prevention of ATP depletion. Preventing the depletion of NAD^+ by PARP preserves the pool of an essential co-factor in the synthesis of ATP. Due in part to this mechanism, PARP inhibition has been shown to be effective in the treatment of diabetes[27], ischemia-reperfusion (I/R) injury[28,29] and IBD[8,30]. And yet, despite a demonstrated efficacy, there exists an understandable hesitancy to interfere with a process as vital as DNA repair. For this reason, while PARP inhibitors have shown tremendous promise in animal models of disease, little to no work has been done with these agents in the clinical setting. Our current study assumes additional importance when considered in light of this fact. We believe that with this work we have delineated a critical mechanism of cellular dysfunction that exists downstream and in reaction to the activation of PARP. It is both striking and intriguing that the pathogenesis of many of the disease states in which PARP inhibition holds promise involve the activation of AMPK, including diabetes[31] and IBD[17]. What is more, there exists a certain poetic sensibility to this signaling cascade as we have delineated it. Inhibiting PARP in a setting where its activity is essential (ie. maintaining genomic integrity) seems counterintuitive; following its activation through to subsequent events, such as AMPK activation may reveal more feasible targets for disease intervention. It is our position that the therapeutic benefit conferred through PARP inhibition is mediated in part by the resultant prevention of AMPK activation. And thus, this study achieves two important objectives: first, by refining the molecular basis of PARP-dependent cellular dysfunction to include AMPK activation we have revealed a potential target for therapeutic intervention that would not compromise the DNA repair machinery, and second, our work implicates chronic AMPK activation as a potentially pathophysiological factor in many disorders in which PARP is involved.

2.5. REFERENCES

1. Berg, D.J., Davidson, N., Kuhn, R., Muller, W., Menon, S., Holland, G., Thompson-Snipes, L., Leach, M.W. & Rennick, D. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest* **98**, 1010-20 (1996).
2. Targan, S.R., Hanauer, S.B., van Deventer, S.J., Mayer, L., Present, D.H., Braakman, T., DeWoody, K.L., Schaible, T.F. & Rutgeerts, P.J. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N Engl J Med* **337**, 1029-35 (1997).
3. van Dullemen, H.M., van Deventer, S.J., Hommes, D.W., Bijl, H.A., Jansen, J., Tytgat, G.N. & Woody, J. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). *Gastroenterology* **109**, 129-35 (1995).
4. Tuzun, A., Erdil, A., Inal, V., Aydin, A., Bagci, S., Yesilova, Z., Sayal, A., Karaeren, N. & Dagalp, K. Oxidative stress and antioxidant capacity in patients with inflammatory bowel disease. *Clin Biochem* **35**, 569-72 (2002).
5. Grisham, M.B. Oxidants and free radicals in inflammatory bowel disease. *Lancet* **344**, 859-61 (1994).
6. Krieglstein, C.F., Cerwinka, W.H., Laroux, F.S., Salter, J.W., Russell, J.M., Schuermann, G., Grisham, M.B., Ross, C.R. & Granger, D.N. Regulation of murine intestinal inflammation by reactive metabolites of oxygen and nitrogen: divergent roles of superoxide and nitric oxide. *J Exp Med* **194**, 1207-18 (2001).
7. Pearson, D.C., Jourd'heuil, D. & Meddings, J.B. The anti-oxidant properties of 5-aminosalicylic acid. *Free Radic Biol Med* **21**, 367-73 (1996).
8. Jijon, H.B., Churchill, T., Malfair, D., Wessler, A., Jewell, L.D., Parsons, H.G. & Madsen, K.L. Inhibition of poly(ADP-ribose) polymerase attenuates inflammation in a model of chronic colitis. *Am J Physiol Gastrointest Liver Physiol* **279**, G641-51 (2000).
9. Le Rhun, Y., Kirkland, J.B. & Shah, G.M. Cellular responses to DNA damage in the absence of Poly(ADP-ribose) polymerase. *Biochem Biophys Res Commun* **245**, 1-10 (1998).
10. Szabo, C. & Dawson, V.L. Role of poly(ADP-ribose) synthetase in inflammation and ischaemia-reperfusion. *Trends Pharmacol Sci* **19**, 287-98 (1998).
11. Beneke, S., Diefenbach, J. & Burkle, A. Poly(ADP-ribosyl)ation inhibitors: promising drug candidates for a wide variety of pathophysiologic conditions. *Int J Cancer* **111**, 813-8 (2004).
12. Kennedy, M., Denenberg, A.G., Szabo, C. & Salzman, A.L. Poly(ADP-ribose) synthetase activation mediates increased permeability induced by peroxynitrite in Caco-2BBe cells. *Gastroenterology* **114**, 510-8 (1998).

13. Madsen, K.L., Malfair, D., Gray, D., Doyle, J.S., Jewell, L.D. & Fedorak, R.N. Interleukin-10 gene-deficient mice develop a primary intestinal permeability defect in response to enteric microflora. *Inflamm Bowel Dis* **5**, 262-70 (1999).
14. Hardie, D.G., Scott, J.W., Pan, D.A. & Hudson, E.R. Management of cellular energy by the AMP-activated protein kinase system. *FEBS Lett* **546**, 113-20 (2003).
15. Carling, D., Clarke, P.R., Zammit, V.A. & Hardie, D.G. Purification and characterization of the AMP-activated protein kinase. Copurification of acetyl-CoA carboxylase kinase and 3-hydroxy-3-methylglutaryl-CoA reductase kinase activities. *Eur J Biochem* **186**, 129-36 (1989).
16. Woods, A., Johnstone, S.R., Dickerson, K., Leiper, F.C., Fryer, L.G., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M. & Carling, D. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol* **13**, 2004-8 (2003).
17. Walker, J., Jijon, H.B., Churchill, T., Kulka, M. & Madsen, K.L. Activation of AMP-activated protein kinase reduces cAMP-mediated epithelial chloride secretion. *Am J Physiol Gastrointest Liver Physiol* **285**, G850-60 (2003).
18. Walker, J., Jijon, H.B., Diaz, H., Salehi, P., Churchill, T. & Madsen, K.L. 5-aminoimidazole-4-carboxamide riboside (AICAR) enhances GLUT2-dependent jejunal glucose transport: a possible role for AMPK. *Biochem J* **385**, 485-91 (2005).
19. Choi, S.L., Kim, S.J., Lee, K.T., Kim, J., Mu, J., Birnbaum, M.J., Soo Kim, S. & Ha, J. The regulation of AMP-activated protein kinase by H(2)O(2). *Biochem Biophys Res Commun* **287**, 92-7 (2001).
20. Watson, A.J., Askew, J.N. & Benson, R.S. Poly(adenosine diphosphate ribose) polymerase inhibition prevents necrosis induced by H2O2 but not apoptosis. *Gastroenterology* **109**, 472-82 (1995).
21. Davies, S.P., Sim, A.T. & Hardie, D.G. Location and function of three sites phosphorylated on rat acetyl-CoA carboxylase by the AMP-activated protein kinase. *Eur J Biochem* **187**, 183-90 (1990).
22. Hallows, K.R., Kobinger, G.P., Wilson, J.M., Witters, L.A. & Foskett, J.K. Physiological modulation of CFTR activity by AMP-activated protein kinase in polarized T84 cells. *Am J Physiol Cell Physiol* **284**, C1297-308 (2003).
23. Hallows, K.R., Raghuram, V., Kemp, B.E., Witters, L.A. & Foskett, J.K. Inhibition of cystic fibrosis transmembrane conductance regulator by novel interaction with the metabolic sensor AMP-activated protein kinase. *J Clin Invest* **105**, 1711-21 (2000).
24. Southan, G.J. & Szabo, C. Poly(ADP-ribose) polymerase inhibitors. *Curr Med Chem* **10**, 321-40 (2003).
25. Jagtap, P. & Szabo, C. Poly(ADP-ribose) polymerase and the therapeutic effects of its inhibitors. *Nat Rev Drug Discov* **4**, 421-40 (2005).
26. McCullough, L.D., Zeng, Z., Li, H., Landree, L.E., McFadden, J. & Ronnett, G.V. Pharmacological inhibition of AMP-activated protein kinase provides neuroprotection in stroke. *J Biol Chem* **280**, 20493-502 (2005).

27. Suarez-Pinzon, W.L., Mabley, J.G., Power, R., Szabo, C. & Rabinovitch, A. Poly (ADP-ribose) polymerase inhibition prevents spontaneous and recurrent autoimmune diabetes in NOD mice by inducing apoptosis of islet-infiltrating leukocytes. *Diabetes* **52**, 1683-8 (2003).
28. Szabados, E., Literati-Nagy, P., Farkas, B. & Sumegi, B. BGP-15, a nicotinic amidoxime derivate protecting heart from ischemia reperfusion injury through modulation of poly(ADP-ribose) polymerase. *Biochem Pharmacol* **59**, 937-45 (2000).
29. Martin, D.R., Lewington, A.J., Hammerman, M.R. & Padanilam, B.J. Inhibition of poly(ADP-ribose) polymerase attenuates ischemic renal injury in rats. *Am J Physiol Regul Integr Comp Physiol* **279**, R1834-40 (2000).
30. Zingarelli, B., Szabo, C. & Salzman, A.L. Blockade of Poly(ADP-ribose) synthetase inhibits neutrophil recruitment, oxidant generation, and mucosal injury in murine colitis. *Gastroenterology* **116**, 335-45 (1999).
31. Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M.F., Goodyear, L.J. & Moller, D.E. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* **108**, 1167-74 (2001).

2.6. EXPERIMENTAL RESULTS

Figure 2.1. PJ34 pre-treatment prevents poly(ADP-ribosyl)ation in oxidatively stressed HT-29 intestinal epithelial cells

HT-29 intestinal epithelial cell monolayers were treated with an increasing dose concentration of the PARP inhibitor PJ34 (0-1.0 μM) for 30 min prior to treatment with a fixed concentration of H_2O_2 (5 mM) for variable duration (0-30 min). Following this incubation, cells were collected in lysis buffer and prepared for SDS-PAGE and subsequent Western blot analysis. The PVDF membrane was probed for poly-ADP ribose (PAR) as a measure of PARP activity.

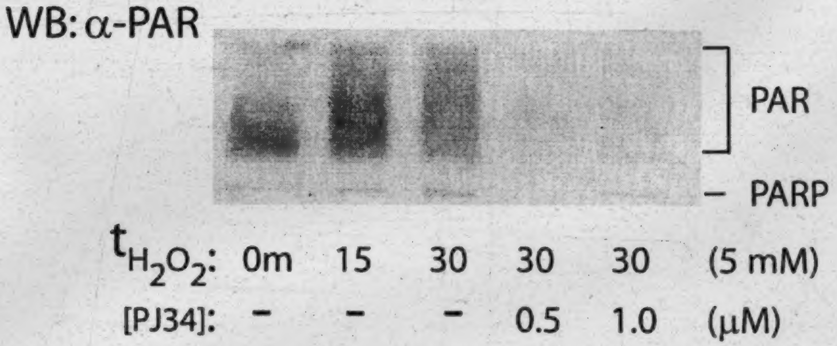

WB: α-PAR
PAR
PARP
$t_{H_2O_2}$: 0m 15 30 30 30 (5 mM)
[PJ34]: - - - 0.5 1.0 (μM)

Figure 2.2.A. PARP inhibition prevents oxidant-dependant AMPK activity

HT-29 intestinal epithelial cell monolayers were treated with an increasing dose concentration of H_2O_2 (0-5 mM) for 30 min, with and without a pretreatment with the PARP inhibitor PJ34 (10 μM, 30 min). Following this incubation, cells were collected in lysis buffer and prepared for SDS-PAGE and subsequent Western blot analysis. The PVDF membrane was probed with an antibody raised against the phosphorylated form of acetyl CoA carboxylase (pACC) as a measure of AMPK activity.

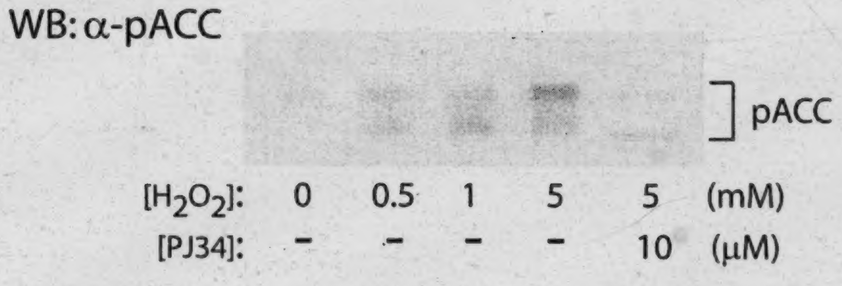


Figure 2.2.B. PARP inhibition prevents oxidant-dependent AMPK activity

HT-29 intestinal epithelial cell monolayers were treated with a fixed concentration of H_2O_2 (1 mM) for 30 min, with and without a pretreatment with the PARP inhibitor 3-AB or the inactive 3-AB analogue ABA (2 mM, 30 min). Following this incubation, cells were collected in lysis buffer and prepared for immunoprecipitation and subsequent assay of AMPK activity. Activity is reported as radioactive decay (counts per minute, cpm) and is indicative of the level of $[^{32}P]$-γ-phosphorylation of the synthetic AMPK substrate SAMS peptide.

$^{*}p<0.05$ vs untreated group, $^{\#}p<0.05$ vs H_2O_2-treated group.

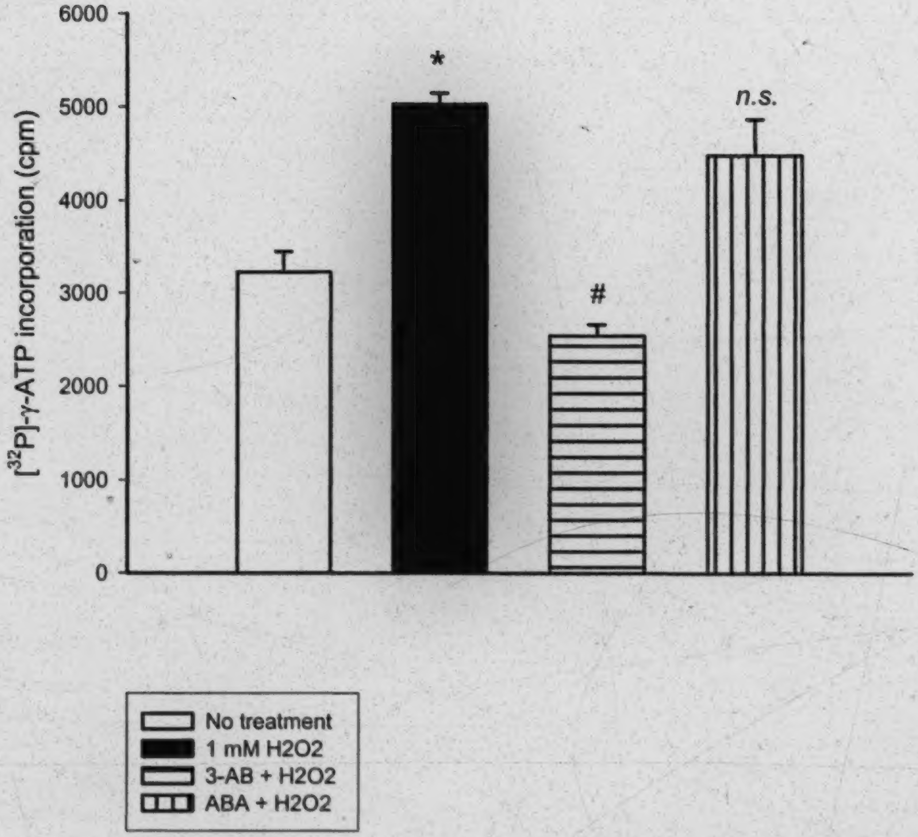
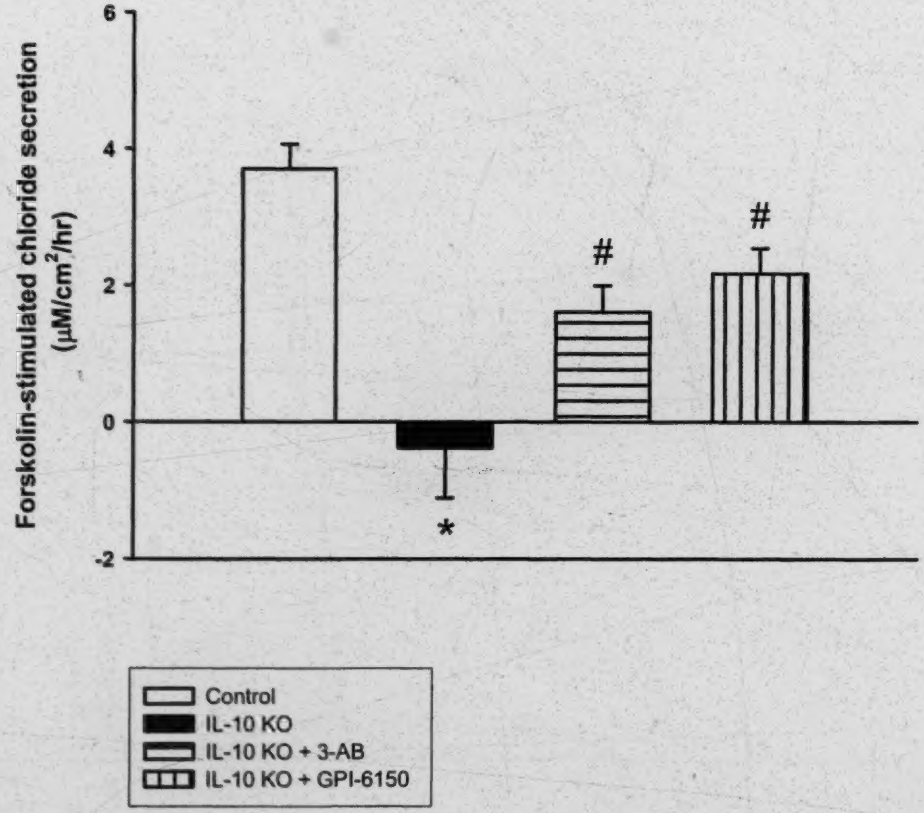


Figure 2.3. PARP inhibition restores a defect in cAMP-dependent colonic chloride secretion in the IL-10 deficient mouse

Treatment of colonic tissue with the PARP inhibitors GPI-6150 (100 μM) and 3-AB (20 mg/kg) was by *in vitro* treatment of bowel explant or by intraperitoneal injection, respectively. cAMP-dependent chloride secretion was measured in Ussing chambers with the reported values representing the measured difference between basal serosal-to-mucosal chloride flux and stimulated (forskolin, 10^{-5} M) serosal-to-mucosal flux. Reported values (μM/cm^2/hr) were calculated from measured radioactive decay, and are indicative of the transport (secretion) of ^{36}Cl by the colonic epithelium. *$p<0.05$ vs wild-type group, $^{\#}p<0.05$ vs IL-10 KO group.



CHAPTER 3

AMP-activated protein kinase is a positive regulator of poly(ADP-ribose) polymerase-1

3.1. INTRODUCTION

Peutz-Jeghers syndrome (PJS) is an autosomal dominantly conferred disorder that is typified by pigmentation of the mucous membranes and multiple hamartomatous polyps in the gastrointestinal tract (benign tumours containing different tissue types)[1,2]. Furthermore, PJS patients are at great risk of developing malignant tumours, predominantly in the intestine but also in other tissues including breast, lung, uterus and ovary. Estimates of the risk to PJS patients of developing some form of cancer (gastrointestinal and nongastrointestinal) range as high as 93%[3].

Germline mutations of the LKB1 gene, located on chromosome 19p13.3 have been identified in the majority of PJS patients[4]. The gene product, also referred to as LKB1, is a multitasking serine-threonine kinase involved in numerous and disparate cellular processes. Recently, LKB1 has been ascribed two critical roles which begin to hint at how a loss of function might contribute to carcinogenesis. First, LKB1 has been demonstrated to play an important part in the maintenance of epithelial cellular polarity[5], and second, LKB1 was recently revealed to be the elusive upstream regulator of the AMP-activated protein kinase (AMPK)[6]. Both of these reports provide insight into possible mechanisms of tumorigenesis in PJS. There has been a ready acceptance that impairment in the polarisation of the intestinal epithelium, such as is seen with the loss of functional LKB1, could contribute to the transformed phenotype of the intestinal epithelium seen in PJS[5]. An alternative and perhaps complementary mechanism for the pathogenesis of PJS may lie with the regulation of AMPK by LKB1. AMPK has been described as a fuel guage of the cell, responding to a variety of metabolic stressors that "tip" the balance in the cellular AMP:ATP ratio. In response to an elevation in this ratio AMPK phosphorylates and downregulates anabolic pathways while inducing catabolic pathways[7]. This redirection of cellular metabolism towards the generation of ATP would be expected to have the parallel effect of inhibiting such processes as energy-requiring macromolecular synthesis, and by extension might negatively regulate cell division. Thus, it has been postulated that cells in which LKB1 function has been lost might possess a proliferative bias[8]. The negative regulation of protein synthesis by AMPK is, as far as is known, by phosphorylation of TSC2, a negative regulator of mammalian target of rapamycin (mTOR)[9], and by extension, LKB1 similarly has a negative regulatory effect on the mTOR pathway[10].

Our findings extend the LKB1-AMPK-carcinogenesis axis by introducing a novel AMPK substrate which we believe to be a contributing factor in the pathogenesis of PJS. Here we describe the positive regulation of the nuclear protein poly(ADP-ribose) polymerase (PARP) by AMPK. This finding is particularly exciting given the abundant evidence linking PARP with DNA repair, chromatin structure and the distribution of genetic material to daughter cells[11]. It has been shown that PARP binds single- and double-strand DNA breaks and is involved in the recruitment of DNA repair elements functioning in base excision repair[12,13]. Thus, in the physiological setting of the intestine, where dietary, bacterial and immune cell -derived genotoxic stressors are abundant, one could surmise that a loss of function of LKB1 might be translated into a deficiency in DNA repair through an inhibited AMPK signal to PARP.

3.2. EXPERIMENTAL PROCEDURES

Cell Culture and Treatments

HT-29 cells were obtained from American Type Culture Collection (Rockwell, MA) and cultured in RPMI 1640 (Gibco, Burlington, ON) supplemented with 10% heat inactivated fetal calf serum (Cansera, Rexdale, ON), 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 2% sodium bicarbonate, 1X penicillin-streptomycin (Gibco, Burlington, ON) and 10 mmol/L HEPES. Confluent monolayers (passage 30-45) were used in all experiments. Where indicated, cells were infected for 18 hr with adenoviral vectors at a multiplicity of infection (MOI) of 50, or as otherwise indicated in figure legends. The Ad5GFP vector encoding for green fluorescent protein was used as a viral negative control. The adenoviruses were washed off and cells returned to growth media for subsequent experimentation.

Western blotting

For Western blot analysis, HT-29 cells were lysed in Mono Q buffer (50 mM β-glycerophosphate, 1 mM EGTA, 0.5 % Triton X-100, 2 mM $MgCl_2$) and cleared by centrifugation, and 50 μg of total cellular lysate protein subjected to electrophoresis on 10% SDS-polyacrylamide gels. Anti-PADPR (abcam, Cambridge, MA), anti-AMPK (Cell Signaling, Beverly, MA) and two anti-PARP (Cell Signaling, Beverly, MA and Upstate Biotechnology, Charlottesville, VA) antibodies were used to detect immunoreactive poly ADP-ribose, total and adenoviral AMPK and full-length and cleaved PARP, respectively using an enhanced chemiluminescence light-detecting kit (Amersham, Arlington Heights, IL). In order to confirm equal loading of protein, all Western blots were stained with Ponceau S.

Immunoprecipitation

For co-immunoprecipitation analysis HT-29 cells were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, Na_3VO_4, 1mM NaF, 0.25% Na-deoxycholate, 1% NP-40, 1 μg/mL aprotinin, leupeptin, pepstatin) and cleared by centrifugation. The supernatant fraction was precleared with protein-G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr. The lysate was incubated with anti-PARP antibody (Upstate Biotechnology, Charlottesville, VA, 10 μg/mg protein) overnight at $4^{\circ C}$ followed by immunoprecipitation with 50 μL protein-G agarose beads (50:50 slurry). The beads were washed and bound proteins eluted by boiling of samples (5m) in 50 μL protein sample buffer (2% β–mercaptoethanol, 10% glycerol, 4% SDS, 100 mM Tris-HCl, trace bromophenol blue, pH 6.8). Immunoprecipititated proteins were resolved by SDS-PAGE and transferred to PVDF. Blots were probed using the immunoprecipitating antibody as well as others as indicated.

Cell-free AMPK assay

The cell-free AMPK assay was performed using purified AMP-activated protein kinase (Upstate Biotechnology) and either SAMS peptide (HMRSAMSGLHLVKRR, Alberta Peptide Institute, Edmonton, AB) or purified, recombinant PARP (Trevigen) as substrate. The kinase reaction was performed according to (Upstate Biotechnology) manufacturer's directions. Briefly, purified enzyme (at concentrations as indicated in figure 3, where 1 unit = 1 nmole phosphate incorporated into 200 μM SAMS peptide/min) was diluted in reaction buffer (20 mM HEPES-NaOH, pH 7.0, 0.4 mM DTT, 0.01% Brij-35, ± 300 μM AMP) containing 1μCi/μL [γ-^{32}P] ATP (complexed with 5 mM $MgCl_2$ and 200 μM unlabelled ATP). SAMS peptide was used at a concentration of 100 μM, while PARP was substituted as substrate at a concentration equivalent to 2 units (where 1 unit is defined as the amount of enzyme required to incorporate 100 pmole of poly(ADP) from NAD into acid-insoluble form in 1 minute). Once prepared, samples were incubated for 15 min at $30^{\circ C}$. Where SAMS peptide was used as kinase substrate samples were spotted on P81 filter papers and washed

3X in 0.75% phosphoric acid, 1X with acetone and allowed to dry prior to immersion in scintillation cocktail. Where PARP was used as substrate, sample proteins were resolved by SDS-PAGE. Incorporation of radio-label into SAMS peptide was determined using a Beckman-coulter scintillation counter, and where PARP was substituted as substrate, a typhoon 8600 phosphorimager (Amersham Biosciences, Piscataway, NJ).

PARP cELISA

The detection of poly(ADP-ribose) polymerase activation by cell ELISA (cELISA) is described in detail by Bakondi et al.[14] Briefly, HT-29 cells were seeded in a 96-well tissue culture plate, and 18 hr prior to beginning the cELISA infected with appropriate adenoviral vectors (see Cell Culture and Treatments, above). Cells were incubated 1 hr ($37^{\circ C}$) in PARP reaction buffer (56 mM HEPES, 28 mM KCl, 28 mM NaCl, 2 mM $MgCl_2$, 0.01% digitonin and 10 μM biotinylated NAD^+ (Trevigen). Following incubation, monolayers were washed 1X with PBS and fixed with ice-cold 95% EtOH for 30 min ($-20^{\circ C}$). Monolayers were washed 1X with PBS and blocked 1 hr ($37^{\circ C}$) with 1% BSA. Block was aspirated and replaced with streptavidin-HRP (1:20000) and incubated 30 min ($37^{\circ C}$). Monolayers were washed 3X with PBS and TMBS added. After 15 min incubation (RT) the reaction was stopped with the addition of 1M H_2SO_4, and absorbance determined at 450 nm.

bioNAD Assay

The biotinylated NAD assay for the detection of PARP auto-poly(ADP-ribosyl)ation is described in detail by Putt et al.[15] Briefly, PARP-1 enzyme (Trevigen, Gaithersburg, MD) is immoblized in a 96 well EIA/RIA plate (Costar, Corning, NY) in a dilution buffer (50 mM Tris-HCl, pH 8.0, 20 μM $ZnCl_2$, 4 mM $MgCl_2$) and incubated overnight ($4^{\circ C}$). Following incubation wells are washed 3X with PBS and incubated 2 hr ($4^{\circ C}$) in reaction buffer (dilution buffer supplemented with 1 mM DTT, 100 μM NAD, 25 μM biotinylated NAD (Trevigen) ± 12.5 μg/mL sheared DNA, ± treatments, as indicated. Following this incubation, wells are washed 3X with PBS and incubated 1hr ($37^{\circ C}$) with streptavidin-HRP (1:20 000). Following this incubation, wells are washed 3X and TMBS added. After 15 min incubation (RT) the reaction was stopped with the addition of 1M H_2SO_4, and absorbance determined at 450 nm.

Statistical Analysis

Data are expressed as means ± SE of ≥ triplicate samples. Statistical significance was assessed using ANOVA, and where appropriate, post-hoc analysis (LSD), (Sigmastat, Systat Software, Inc., Point Richmond, CA).

3.3. RESULTS

We began our investigation by examining the regulation of PARP by AMPK in HT-29 intestinal epithelial cells. The catalytic action of PARP involves the poly-ADP ribosylation of various nuclear proteins, including PARP itself, a process termed automodification[16]. The automodification of PARP is readily apparent in response to treatment with such genotoxins as H_2O_2, and can be visualized by immunoblot analysis using an antibody raised against ADP-ribose polymers. We over-expressed constitutively active AMPK (Ad.CA-AMPK) in HT-29 cells, using an adenoviral gene delivery approach in order to examine if PARP is regulated by AMPK. Cells infected with Ad.CA-AMPK demonstrated an MOI-dependent increase in PARP automodification as compared with control cells expressing green fluorescent protein (Ad.GFP, Figure 3.1.A). Continuing along this line of investigation, we next infected HT-29 cells with the same viral titres as previously mentioned and assessed PARP activity in a cell-based ELISA, or cELISA. Briefly, the PARP cELISA assays for the incorporation of biotinylated NAD (bioNAD) by PARP (and other PARP substrate proteins) in the ADP-ribosylation reaction[14]. The results from this experiment confirmed our initial Western-blot findings, with Ad.CA-AMPK resulting in a significant (≈ 2.8 fold) increase in bioNAD incorporation versus the uninfected epithelial cells (Figure 3.1.B). A dose-dependent increase in PARP activation in response to treatment with the genotoxin H_2O_2 is included as a positive control. Of note, infection with the Ad.GFP also increases bioNAD incorporation, presumably through indirect means, and likely as an artifact of viral infection. Nonetheless, the increase in assay activity in that control group raised the question of whether the infection process might be a contributing factor to the observed increase in PARP activity. Accordingly, our subsequent experiments were designed to evaluate whether the regulatory relationship between AMPK and PARP was being mediated through direct interaction. We examined the possibility that the two proteins shared a physical interaction by immunoprecipitation Western-blot analysis. In this experiment HT-29 cells were treated with an increasing concentration of H_2O_2 and lysates were prepared for immunoprecipitation. We included a treatment with H_2O_2 to determine whether the regulation of PARP by AMPK was genotoxin-dependent (H_2O_2 is an activator of both PARP and AMPK), and the inclusion of this parameter to the experiment had the added effect of ensuring that PARP was in fact the immunoprecipitate in our pull-down, as we were also able to immunoblot against automodified PARP, or PAR (Figure 3.2). The results from this experiment revealed two important pieces of data, the first being the recognition of AMPK and PARP as co-immunoprecipitates, a strong indication that these proteins interact physically within the cell, and the second being that the interaction between PARP and AMPK likely occurs in the resting cell, and not solely as a stress response to exogenous stimuli, as co-immunoprecipitation of the proteins was evident irrespective of treatment with H_2O_2.

With good evidence that the regulation of PARP by AMPK involved their physical interaction we next focused our investigation towards the nature of that interaction. Specifically, we asked the question 'does regulation of PARP activity by AMPK involve kinase activity?' To address this question we developed an in vitro kinase assay employing purified, recombinant PARP and AMPK. Figure 3.3.A demonstrates the specificity of the assay using an engineered AMPK substrate, SAMS peptide. As is routinely reported, the inclusion of the AMPK activator AMP (300 μM) increased kinase activity by two to three times. When we substituted purified, recombinant PARP for SAMS peptide as substrate, the results from this assay were unambiguous; increasing phosphorylation of PARP was evident through an increasing range of concentrations of AMPK (Figure 3.3.B). Furthermore, phosphorylation of PARP was increased when the assay conditions were modified to include the canonical AMPK activator AMP (300 μM, Figure 3.3.B). This data served not only to reinforce our earlier findings, but was interesting in its own regard. So far as we are aware, this report details the first instance of kinase regulation of PARP. As mentioned, the putative activator of PARP is DNA strand breakage, but for a protein to escape all manner of kinase-dependent regulation seems unlikely. However, there have been no reports in the literature of such regulation. The consensus phosphorylation site for AMPK is highly degenerate, and it has been estimated that as many as 50% of the constituents of the human proteome may serve as substrates. In light of this fact, it is rather less remarkable that the regulation of PARP by AMPK seems to involve phosphorylation. Our lines of investigation had by this point led us to believe that AMPK and PARP shared a direct, functional association in intestinal epithelial cells. To reconfirm this finding we modified an enzymatic assay for PARP[15] to assess the functional consequences of AMPK regulation in an *in vitro* system. In a similar fashion to the PARP cELISA, the assay utilizes the incorporation of bioNAD and a subsequent streptavidin-HRP binding reaction to assess enzyme activity. In our experiment we immobilized PARP on a high-binding 96 well plate and incubated the enzyme with and without AMPK, and in the presence and absence of ATP (125 μM). As with previous findings, our results clearly indicate that the regulation of PARP by AMPK is of a positive nature, as the incorporation of bioNAD by PARP was dramatically increased with the inclusion of AMPK (Figure 3.3.C). Using sheared salmon-sperm DNA as a positive control we are able to demonstrate a further two-fold increase in activity over control values, but the increase in activity is only observable upon the inclusion of ATP in the reaction buffer. We take this finding to be further indication that PARP is a substrate for AMPK phosphorylation, and that this phosphorylation dramatically increases PARP activity.

The finding that AMPK positively regulates PARP activity is somewhat contradictory to what one might expect the relationship between these two proteins to entail. In fact, it seems paradoxical that AMPK would promote PARP activity, given that a recognized endpoint to PARP activation is the depletion of cellular ATP. At the cellular level, however, the interaction between AMPK and PARP can be viewed from a more holistic viewpoint. While the chief mandate for AMPK is the preservation and/or restoration of cellular ATP levels, to accomplish this task at the expense of maintaining genomic integrity would seem counterintuitive. Therefore, while the primary concern of this study was to report the ramifications of our findings within the context of PJS, it has not escaped our notice that perhaps AMPK is poised within the cell to act as a molecular switch, capable of modulating the cellular response between survival and cell death.

Jones et al.[17] have very recently reported findings that would indicate this to be the case. In that study the authors report that AMPK induces a p53-dependent metabolic checkpoint, with persistent activation of AMPK leading to accelerated p53-dependent cellular senescence. Within this context, AMPK can be viewed as a cell-intrinsic regulator of replicative cell division that coordinates cellular proliferation with energy availability. To further investigate that end, we employed the pharmacological activator of AMPK 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR, 1mM) to assess the impact of sustained AMPK activation in HT-29 epithelial cells. By Western blot analysis we were able to demonstrate the cleavage of PARP in these cells in a manner that increased with the duration of AICAR treatment (Figure 3.4). PARP cleavage is a classic marker of caspase-dependent apoptosis. The involvement of AMPK in the induction of apoptosis has been reported in other cell types, including hepatocytes[18] and pancreatic beta cells[19]. Interestingly, and consistent with our observation in intestinal epithelial cells, both of these reports specify a requirement for sustained or prolonged AMPK activation in the induction of regulated cell death. The notion that AMPK acts acutely to “rescue” the cell from ATP-depletion and subsequent cell death is in keeping with its role as described extensively in the literature. However, sustained activation of the kinase, presumably accompanied by the resultant long-term inhibition of transcription and protein synthesis, would undoubtedly be deleterious to the cell, and at that point apoptosis over necrosis would be the preferred outcome. Returning to the context of PJS, it also may be the case that an early event in the development of intestinal polyposis and carcinogenesis may involve impairment in AMPK-dependent apoptosis, resulting from the absence of LKB1 signalling. PARP stimulation is an apoptotic event that precedes PARP cleavage; it is conceivable that AMPK participates in the stimulatory process and that, in the absence of this stimulation, the apoptotic cascade is compromised. The question of whether AMPK activity is required for apoptosis remains to be answered, and is a focus of our ongoing studies.

3.4. DISCUSSION

In recent years a number of plausible theories regarding the ramifications of a loss of function of LKB1 have been put forth, and each in their own regard may play a contributing part in the pathogenesis of PJS. Of note, LKB1 seems to be involved in the regulation of epithelial cell polarity[5], energy metabolism[6], and, perhaps apoptosis[20]. Given the recognized role of LKB1 as a master kinase within the cell, it is highly unlikely that a singular mechanism for the development of carcinogenesis in PJS will be distilled. In recognition of that fact, it is nonetheless fascinating to speculate on the role that a PARP deficiency might play within the context of this disease. The development of malignant tumours is a process dependent on the accumulation of genetic and epigenetic alterations in cells. Genomic instability markedly accelerates the carcinogenic process. Within the confines of PJS it is possible that a dysfunction in PARP-dependent DNA repair could be translated to an enhanced rate of tumour formation. Interestingly, a comparison of the rates of spontaneous mutant frequencies in the livers of PARP$^{-/-}$ and PARP$^{+/+}$ mice revealed no significant differences, however, after treatment with an alkylating agent the frequency of mutations was increased significantly in the PARP$^{-/-}$ animals[21]. Furthermore, elevated susceptibility to colon carcinogenesis induced by an alkylating agent in PARP$^{-/-}$ mice has been established[22], and tumours developed in these animals involved an alteration of Wnt-beta-catenin signalling, consistent with previously published results linking LKB1 with this critical oncogenic pathway[23]. These findings are intriguing when considered in light of the pathogenesis of PJS, where a germ-line mutation is first and primarily manifested in the gastrointestinal tract. By virtue of its function the intestine is in constant exposure to a variety of carcinogens that would necessitate efficient DNA repair machinery. Intriguingly, an absence of stimulation of PARP activity in patients predisposed to colon cancer has previously been demonstrated[24].

An important underlying question arises from this study: excessive PARP activation leads to ATP depletion, which serves to activate AMPK, which, as demonstrated, leads back to the potentiation of PARP activity; what is the metabolic basis for this mechanism? Irrespective of its function as a tumour suppressor, the putative role for AMPK is in the restoration of cellular ATP levels. It may be that PARP activation is a means to this end. Oei and Zeigler[25] have reported provocative findings that would suggest that ADP-ribosylation might in fact create a protected, nuclear pool of ATP through the coordinated action of poly(ADP-ribose) glycohydrolase (PARG). There is a dramatic decrease in cellular ATP concentration following exposure to genotoxic agents[26] and it may be that a role of poly(ADP-ribosyl)ation may be to facilitate the extraction of energy from NAD and its immediate use for DNA repair. It is intriguing to speculate that AMPK may play a role in this process; by engaging PARP, AMPK would in effect create a "pool" of ATP, exclusively reserved for the privileged process of DNA repair.

This study is the first to identify the DNA-repair enzyme PARP as a substrate for regulation by AMPK. We propose that the impairment of PARP function by AMPK inhibition is a determinant in the carcinogenic process, and that this mechanism may be important in the pathogenesis of Peutz-Jeghers syndrome.

3.5. REFERENCES

1. Peutz, J.L.A. On a very remarkable case of familial polyposis of the mucuous membrane of the intestinal tract and nasopharynx accompanied by peculiar pigmentation of the skin and mucuous membranes. *Ned Tijdschr Geneeskd* **10**, 134-146 (1921).
2. Jeghers, H., Mckusick, V.A., Karz, K.H. Generalised intestinal polyposis and melanin spots of oral mucosa, lips and digits: a syndrome of diagnostic significance. *New Engl. J. Med.* **241**, 992-1005 (1944).
3. Giardiello, F.M., Brensinger, J.D., Tersmette, A.C., Goodman, S.N., Petersen, G.M., Booker, S.V., Cruz-Correa, M. & Offerhaus, J.A. Very high risk of cancer in familial Peutz-Jeghers syndrome. *Gastroenterology* **119**, 1447-53 (2000).
4. Hemminki, A., Markie, D., Tomlinson, I., Avizienyte, E., Roth, S., Loukola, A., Bignell, G., Warren, W., Aminoff, M., Hoglund, P., Jarvinen, H., Kristo, P., Pelin, K., Ridanpaa, M., Salovaara, R., Toro, T., Bodmer, W., Olschwang, S., Olsen, A.S., Stratton, M.R., de la Chapelle, A. & Aaltonen, L.A. A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. *Nature* **391**, 184-7 (1998).
5. Baas, A.F., Kuipers, J., van der Wel, N.N., Batlle, E., Koerten, H.K., Peters, P.J. & Clevers, H.C. Complete polarization of single intestinal epithelial cells upon activation of LKB1 by STRAD. *Cell* **116**, 457-66 (2004).
6. Woods, A., Johnstone, S.R., Dickerson, K., Leiper, F.C., Fryer, L.G., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M. & Carling, D. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol* **13**, 2004-8 (2003).
7. Carling, D. The AMP-activated protein kinase cascade--a unifying system for energy control. *Trends Biochem Sci* **29**, 18-24 (2004).
8. Spicer, J. & Ashworth, A. LKB1 kinase: master and commander of metabolism and polarity. *Curr Biol* **14**, R383-5 (2004).
9. Bolster, D.R., Crozier, S.J., Kimball, S.R. & Jefferson, L.S. AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. *J Biol Chem* **277**, 23977-80 (2002).
10. Shaw, R.J., Bardeesy, N., Manning, B.D., Lopez, L., Kosmatka, M., DePinho, R.A. & Cantley, L.C. The LKB1 tumor suppressor negatively regulates mTOR signaling. *Cancer Cell* **6**, 91-9 (2004).
11. Masutani, M., Nakagama, H. & Sugimura, T. Poly(ADP-ribose) and carcinogenesis. *Genes Chromosomes Cancer* **38**, 339-48 (2003).
12. Masson, M., Niedergang, C., Schreiber, V., Muller, S., Menissier-de Murcia, J. & de Murcia, G. XRCC1 is specifically associated with poly(ADP-ribose) polymerase and negatively regulates its activity following DNA damage. *Mol Cell Biol* **18**, 3563-71 (1998).
13. Caldecott, K.W., Aoufouchi, S., Johnson, P. & Shall, S. XRCC1 polypeptide interacts with DNA polymerase beta and possibly poly (ADP-

ribose) polymerase, and DNA ligase III is a novel molecular 'nick-sensor' in vitro. *Nucleic Acids Res* **24**, 4387-94 (1996).

14. Bakondi, E., Bai, P., Szabo, E.E., Hunyadi, J., Gergely, P., Szabo, C. & Virag, L. Detection of poly(ADP-ribose) polymerase activation in oxidatively stressed cells and tissues using biotinylated NAD substrate. *J Histochem Cytochem* **50**, 91-8 (2002).
15. Putt, K.S. & Hergenrother, P.J. An enzymatic assay for poly(ADP-ribose) polymerase-1 (PARP-1) via the chemical quantitation of NAD(+): application to the high-throughput screening of small molecules as potential inhibitors. *Anal Biochem* **326**, 78-86 (2004).
16. Yoshihara, K., Hashida, T., Yoshihara, H., Tanaka, Y. & Ohgushi, H. Enzyme-bound early product of purified poly(ADP-ribose) polymerase. *Biochem Biophys Res Commun* **78**, 1281-8 (1977).
17. Jones, R.G., Plas, D.R., Kubek, S., Buzzai, M., Mu, J., Xu, Y., Birnbaum, M.J. & Thompson, C.B. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol Cell* **18**, 283-93 (2005).
18. Meisse, D., Van de Casteele, M., Beauloye, C., Hainault, I., Kefas, B.A., Rider, M.H., Foufelle, F. & Hue, L. Sustained activation of AMP-activated protein kinase induces c-Jun N-terminal kinase activation and apoptosis in liver cells. *FEBS Lett* **526**, 38-42 (2002).
19. Kefas, B.A., Heimberg, H., Vaulont, S., Meisse, D., Hue, L., Pipeleers, D. & Van de Casteele, M. AICA-riboside induces apoptosis of pancreatic beta cells through stimulation of AMP-activated protein kinase. *Diabetologia* **46**, 250-4 (2003).
20. Karuman, P., Gozani, O., Odze, R.D., Zhou, X.C., Zhu, H., Shaw, R., Brien, T.P., Bozzuto, C.D., Ooi, D., Cantley, L.C. & Yuan, J. The Peutz-Jegher gene product LKB1 is a mediator of p53-dependent cell death. *Mol Cell* **7**, 1307-19 (2001).
21. Shibata, A., Kamada, N., Masumura, K., Nohmi, T., Kobayashi, S., Teraoka, H., Nakagama, H., Sugimura, T., Suzuki, H. & Masutani, M. Parp-1 deficiency causes an increase of deletion mutations and insertions/rearrangements in vivo after treatment with an alkylating agent. *Oncogene* **24**, 1328-37 (2005).
22. Nozaki, T., Fujihara, H., Watanabe, M., Tsutsumi, M., Nakamoto, K., Kusuoka, O., Kamada, N., Suzuki, H., Nakagama, H., Sugimura, T. & Masutani, M. Parp-1 deficiency implicated in colon and liver tumorigenesis induced by azoxymethane. *Cancer Sci* **94**, 497-500 (2003).
23. Spicer, J., Rayter, S., Young, N., Elliott, R., Ashworth, A. & Smith, D. Regulation of the Wnt signalling component PAR1A by the Peutz-Jeghers syndrome kinase LKB1. *Oncogene* **22**, 4752-6 (2003).
24. Cristovao, L., Lechner, M.C., Fidalgo, P., Leitao, C.N., Mira, F.C. & Rueff, J. Absence of stimulation of poly(ADP-ribose) polymerase activity in patients predisposed to colon cancer. *Br J Cancer* **77**, 1628-32 (1998).
25. Oei, S.L. & Ziegler, M. ATP for the DNA ligation step in base excision repair is generated from poly(ADP-ribose). *J Biol Chem* **275**, 23234-9 (2000).

26. Sims, J.L., Berger, S.J. & Berger, N.A. Poly(ADP-ribose) Polymerase inhibitors preserve nicotinamide adenine dinucleotide and adenosine 5'-triphosphate pools in DNA-damaged cells: mechanism of stimulation of unscheduled DNA synthesis. *Biochemistry* **22**, 5188-94 (1983).

3.6. EXPERIMENTAL RESULTS

Figure 3.1.A. AMPK is a positive regulator of PARP activity in intestinal epithelial cells

HT-29 intestinal epithelial cells were transduced with an increasing MOI of adenoviruses encoding for a constitutively active AMPK variant (Ad.CA-AMPK) or, as an infection control, GFP (Ad.GFP). Epithelial monolayers were incubated with the indicated viral titres for 18 hr, washed twice with PBS, and incubated for an additional 6 hr in complete media (RPMI 1640 w/ 10% FBS). Following this incubation, cells were collected in lysis buffer and prepared for SDS-PAGE and subsequent Western blot analysis. The PVDF membrane was probed first for poly-ADP ribose (PAR) as a measure of PARP activity and second for AMPK, to visualize the protein product of adenoviral-mediated gene transfer.

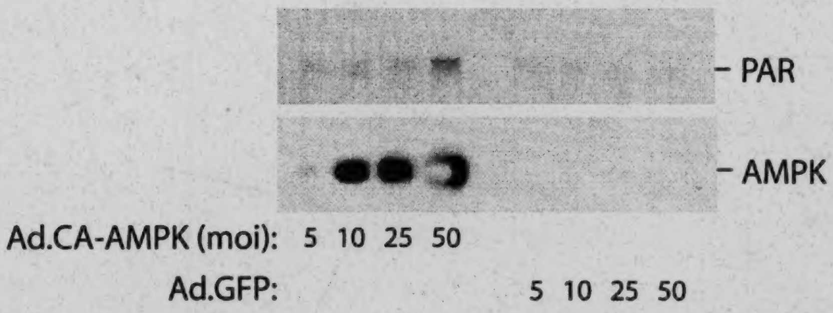


Figure 3.1.B. AMPK is a positive regulator of PARP activity in intestinal epithelial cells

HT-29 cells were transduced as described at a MOI of 50. Following transduction monolayers were incubated with a reaction buffer containing biotinylated NAD (bioNAD) as substrate for ADP-ribosylation reactions. Subsequent to this incubation monlayers were washed with PBS and fixed in 95% ethanol prior to incubation with streptavidin-HRP. Incorporation of bioNAD was quantified between groups by measuring optical density subsequent to incubation with an HRP substrate.
*p<0.01, #p<0.05 vs untreated cells.

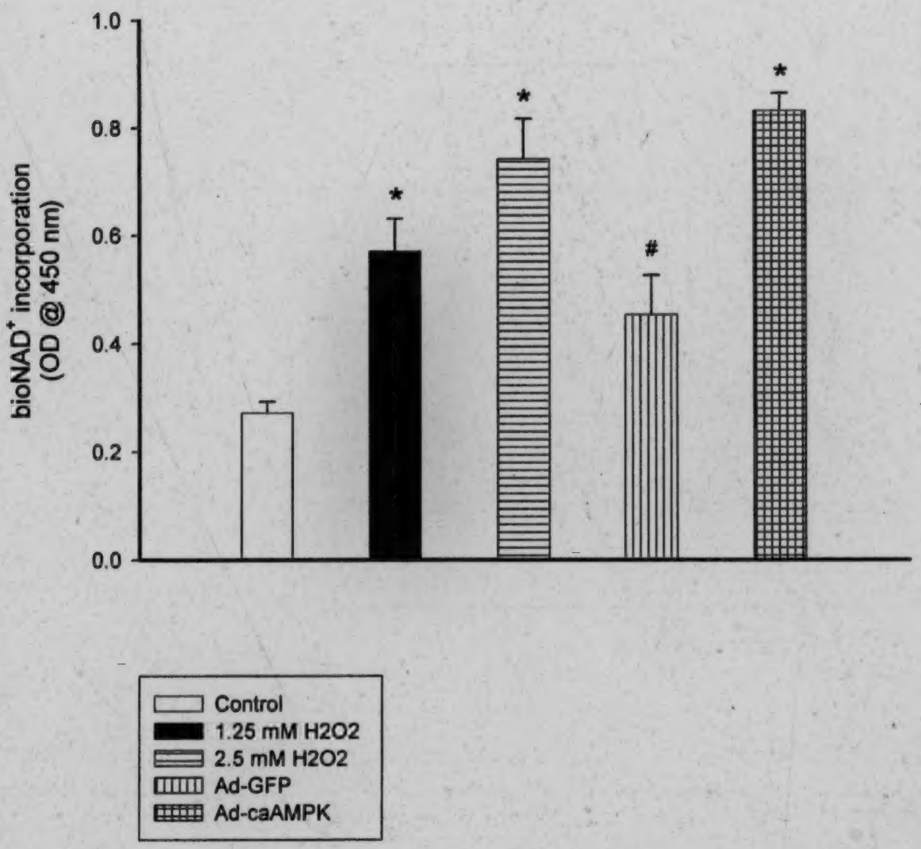


Figure 3.2. AMPK and PARP co-immunoprecipitate from HT-29 intestinal epithelial cell lysate, independent of exogenous activation

HT-29 intestinal epithelial cell monolayers were treated with a fixed concentration of H_2O_2 (5 mM) for 0-60 min and subsequently harvested in a lysis buffer amenable to immunoprecipitation. Protein concentrations between groups were standardized to 2 mg/mL total protein concentration and PARP was immunoprecipitated from the lysate. Samples were subjected to SDS-PAGE and Western-blot analysis. The PVDF membrane was probed first for PARP and subsequently for PAR and AMPK.

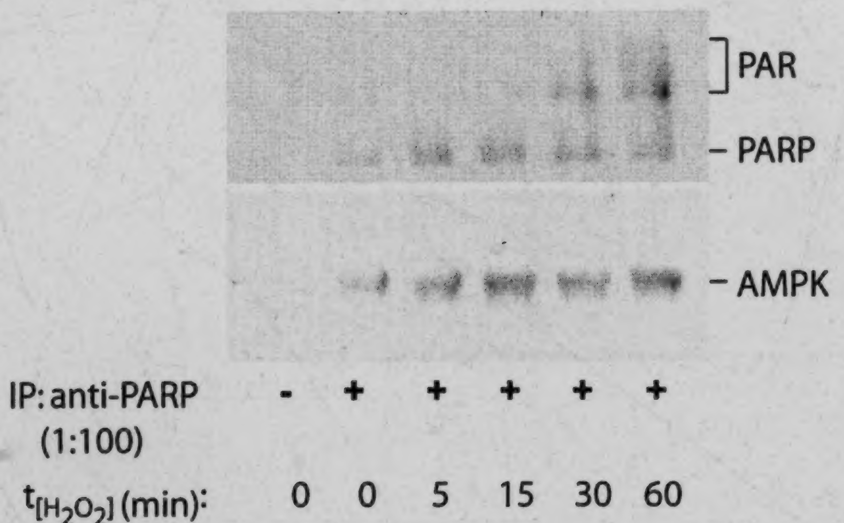


Figure 3.3.A. PARP is a substrate for AMPK-dependent phosphorylation, and activation of AMPK potentiates this reaction

As a positive control for the *in vitro* kinase assay (panel B), purified AMPK in increasing concentrations (0-10 mU/μL) was incubated with the synthetic AMPK substrate SAMS peptide in the presence or absence of 300 μM AMP as activator. Incorporation of [^{32}P]-γ-ATP was assessed by spotting the substrate peptide-containing reaction mixture onto filter-papers and measuring radioactive decay by liquid scintillation counting.

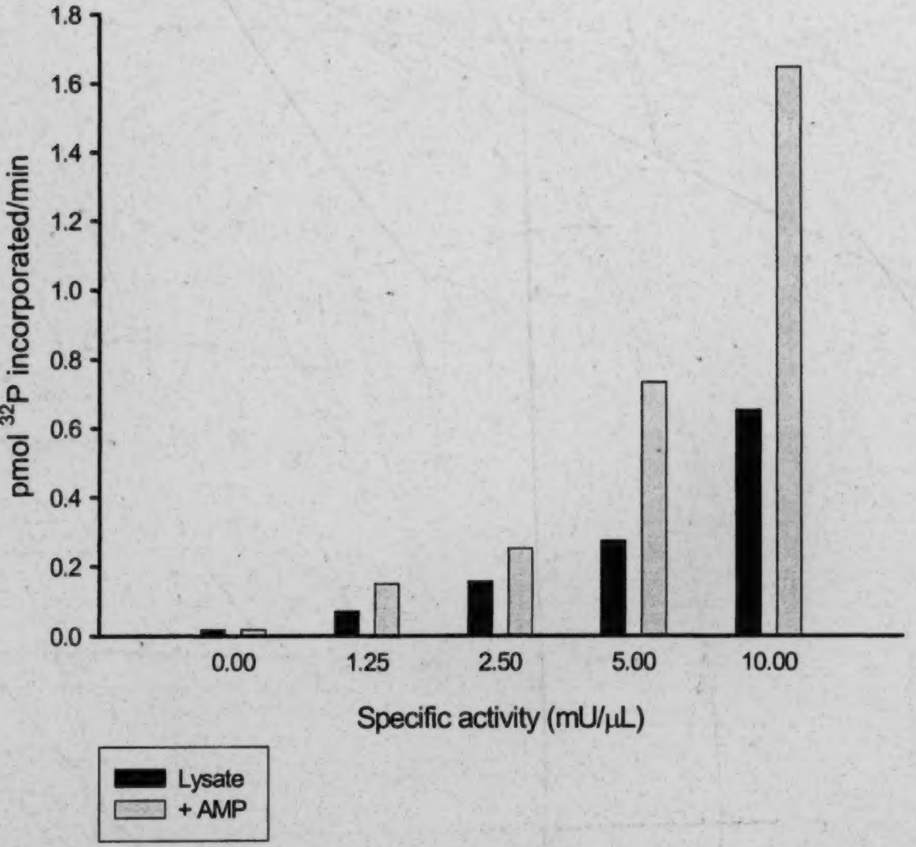


Figure 3.3.B PARP is a substrate for AMPK-dependent phosphorylation, and activation of AMPK potentiates this reaction

The experiment described in panel A was repeated with purified, recombinant PARP substituted as substrate. The unlabelled band at the vertical midpoint of the gel is BSA, included in the reaction mixture as a negative control. Following incubation, the protein mixture was separated by SDS-PAGE and incorporation of [^{32}P]-γ-ATP was visualized using a typhoon phosphoimager. The incorporation of [^{32}P]-γ-ATP was increased with the addition of 300 μM AMP. Phosphorylation of AMPK is also observed under the conditions of the assay.

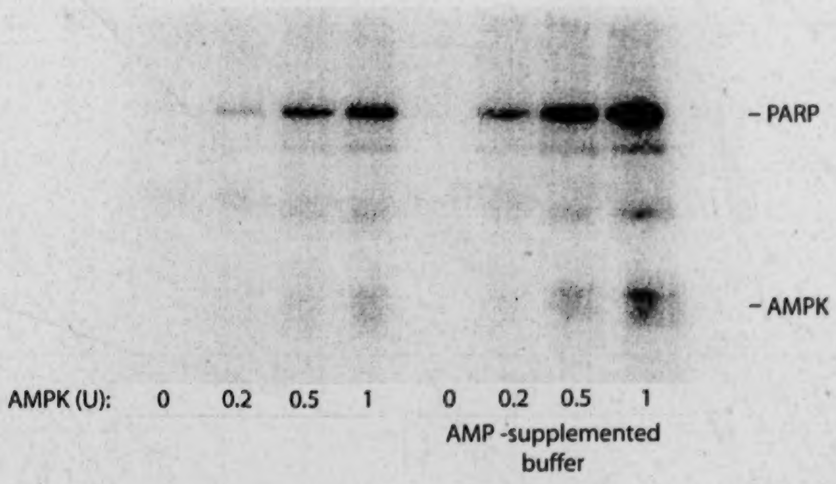


Figure 3.3.C. PARP is a substrate for AMPK-dependent phosphorylation, and activation of AMPK potentiates this reaction

A cell-free enzymatic assay for PARP activity by the chemical quantification of NAD was modified to include purified AMPK. Purified, recombinant PARP was immobilized and combined with sheared DNA as an activator in the presence and absence of 10 mU AMPK. Incorporation of bioNAD by PARP was quantified between groups by measuring optical density subsequent to incubation with an HRP substrate.
*p<0.01, #p<0.05 vs untreated cells.

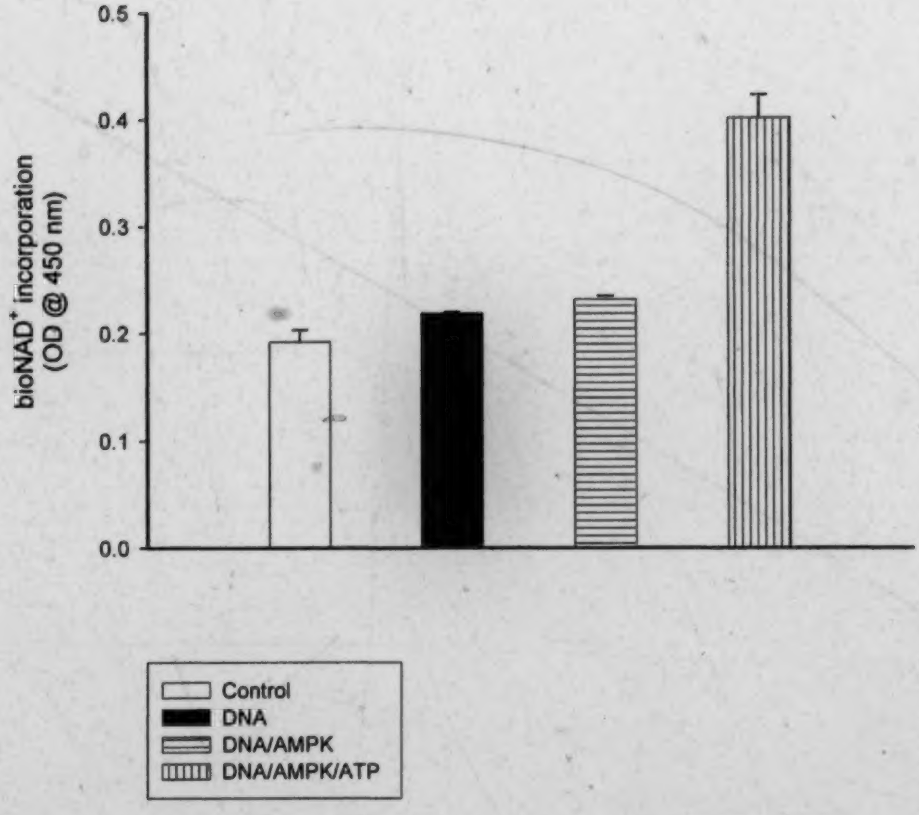
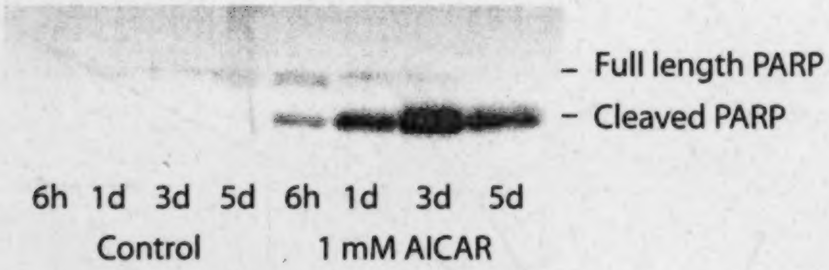


Figure 3.4. Sustained treatment with AICAR induces apoptosis in HT-29 epithelial cells

HT-29 intestinal epithelial cells were treated with AICAR (1 mM) for the time periods indicated (0-5 days) and subsequently harvested in a lysis buffer. Total lysate proteins were separated by SDS-PAGE and analyzed by Western blot. PVDF membranes were probed using an antibody recognizing the full-length PARP protein, as well as the caspase cleavage product.



CHAPTER 4

Activation of AMP-activated protein kinase reduces cAMP-mediated epithelial chloride secretion

Am J Physiol Gastrointest Liver Physiol. 2003 Nov;285(5):G850-60.

4.1. INTRODUCTION

A key feature of intestinal inflammation is the production of oxidative species by immune cells, leading to epithelial cell damage[1]. These oxygen- and nitrogen-derived species react with membrane lipids, intracellular proteins and DNA, resulting in changes to epithelial architecture and function within the gut. Oxidative damage to epithelial cells results in reduced cellular ATP levels, and ultimately, a failure of energy (ATP)-dependent processes. In addition, acute and chronic intestinal inflammation are characterized by high mucosal levels of pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-1β which have also been demonstrated to reduce intracellular ATP levels *in vitro*[2,3]. In the gut, ATP-dependent cellular processes include the maintenance of epithelial barrier function and the active secretion of anions; and indeed, profound alterations in intestinal barrier and transport function are seen both in animal models of inflammation[4-7] and in patients with inflammatory bowel diseases[8-10].

Recently, AMP-activated protein kinase (AMPK) has emerged as a sensor of energy stores within the cell[11,12]. AMPK is a serine/threonine kinase that exists as a heterotrimer composed of a catalytic α subunit and regulatory β and γ subunit[13,14]. AMPK activity increases during conditions of metabolic stress as a result of an elevated intracellular AMP:ATP ratio[15,16]. Intracellular metabolic stress is seen under varied conditions, including heat shock[17], hypoglycaemia[18-20], hypoxia[21-23], ischaemia[24,25] and oxidant exposure[26,27]. Activation of AMPK involves an allosteric mechanism by which AMP binds to AMPK, in addition to a phosphorylation of a threonine residue on the catalytic subunit of AMPK catalyzed by an upstream kinase, AMPK kinase (AMPKK)[28]. AMPK responds to alterations in cellular energy changes by regulating both ATP-consuming and ATP-generating pathways[12,15]. Studies have shown AMPK activation to regulate numerous pathways, including the inhibition of fatty acid, triglyceride and sterol synthesis[29,30], and the stimulation of glucose uptake[19,31,32], glycolysis[21,25], and fatty acid oxidation[33]. In addition, AMPK activation has also been linked with changes in the expression of various different genes[34].

In the gut, the cystic-fibrosis transmembrane conductance regulator (CFTR) is the primary channel responsible for chloride secretion[35]. CFTR requires the hydrolysis of ATP for activity, and has been shown to physically interact with AMPK, with activation of AMPK resulting in an inhibition of CFTR in epithelial cells[36,37]. Thus, we hypothesized that the activation of AMPK in response to metabolic stress may be responsible for the dysfunction in chloride secretion seen under conditions of chronic inflammation[4,8,38].

In the present study we examined the role of AMPK in regulating epithelial chloride secretion under chronic inflammatory conditions in colonic tissue from IL-10 deficient mice and *in vitro* in human colonic T_{84} epithelial monolayers. Results obtained in this study suggest that the hyporesponsiveness observed under conditions of chronic inflammation is related to AMPK activation.

4.2. EXPERIMENTAL PROCEDURES

Animals

Homozygous IL-10 deficient mice, generated on a 129 Sv/Ev background, and wild-type 129 Sv/Ev controls were housed behind a barrier under specific pathogen-free conditions. The mice had *ad libitum* access to autoclaved 9% fat rodent blocs and sterile filtered water. The facility's sanitation was verified by Health Sciences Lab Animal Services at the University of Alberta (Edmonton, Alberta, Canada). To demonstrate the role of AMPK in the defect in chloride secretion seen in the IL-10 deficient mice, colonic tissue from IL-10 deficient mice was treated *in vitro* with compound C (6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-ylpyyrazolo[1,5-a]pyrimidine) (75 μM). Compound C is a specific potent reversible inhibitor of AMPK that is competitive with ATP in the absence of AMP[39]. Compound C was kindly provided by Dr. G. Zhou (Merck & Co. Rahway, NJ). Colons from 129 Sv/Ev wild-type mice were treated *in vitro* with the AMPK–specific activator AICAR (5-aminoimidazole-4-carboxamide 1-beta-D-ribofuranoside) (1 mM). AMPK is activated pharmacologically by 5-amino-4-imidazolecarboxamide (AICA) riboside monophosphate (ZMP), which mimics the effects of AMP on the AMPK cascade[28]. All experiments were performed according to the institutional guidelines for the care and use of laboratory animals in research and with the permission of the local ethics committee.

Epithelial Chloride Secretion

Mice were sacrificed by cervical dislocation, and a segment of proximal colon was removed. Colonic tissue was mounted in Lucite chambers exposing mucosal and serosal surfaces to 10 mL of oxygenated Krebs buffer (in mmol/L: 115 NaCl, 8 KCl, 1.25 $CaCl_2$, 1.2 $MgCl_2$, 2 KH_2PO_4, 25 $NaHCO_3$; pH 7.35). When a Cl^- free buffer was used, 115 mmol/L Na^+-gluconate replaced NaCl, 1.2 mmol/L $MgSO_4$ replaced $MgCl_2$, and 1.2 mmol/L Ca^{2+} -gluconate replaced $CaCl_2$. The Cl/HCO_3^- free buffer used was similar except that 125 mmol/L Na-gluconate was used to replace all Cl^- and HCO_3^- was replaced by a 10 mmol/l HEPES buffer titrated to pH 7.4 with 1 mol/l Tris base. Acetazolamide (10^{-3} mol/L) was added to the Cl/HCO_3^- free buffer to inhibit endogenous HCO_3^- production. The Cl/HCO_3^- free buffer was gassed with 100% O_2. The buffers were maintained at 37°C by a heated water jacket and circulated by CO_2/O_2. Fructose (10 mmol/L) was added to the serosal and mucosal sides. The spontaneous transepithelial potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate short-circuit current (Isc) with an automatic voltage clamp (DVC 1000 World Precision Instruments, New Haven, CT), except for 5-10 seconds every 5 minutes when PD was measured by removing the voltage clamp. Tissue ion conductance (G) was calculated from PD and Isc according to Ohm's Law. PD is expressed as millivolts (mV), Isc as microamperes per square centimeter ($\mu A/cm^2$), and G as millisiemens/cm^2 (mS/cm^2). Baseline Isc and G were measured after a 20-minute equilibration period. Increases in Isc were induced by addition of the adenylate cyclase-activating agent forskolin (10^{-8} to 10^{-4} M) to the serosal surface. Epithelial responsiveness was defined as the maximal increase in Isc to occur within 5 minutes of exposure to secretagogues.

For the measurement of basal chloride fluxes, the tissue was clamped at zero voltage by continuously introducing an appropriate Isc with an automatic voltage clamp, except for 5-10 seconds every 5 minutes when PD was measured by removing the voltage clamp. Tissue pairs were matched for conductance and discarded if conductance varied by > 20%. 5 μCi ^{36}Cl was added to the serosal side after mounting and the tissue was allowed to equilibrate for 20 minutes. Unidirectional flux from serosal-to-mucosal surface was determined for paired tissues by measuring four consecutive five-minute fluxes prior to the addition of forskolin (10^{-4} M) and two five-minute fluxes following the addition of the secretagogue.

Chloride flux in response to forskolin is reported as the difference between the averaged values of the four consecutive five-minute fluxes prior to the addition of the secretagogue and the averaged values of the two consecutive five-minute fluxes post-stimulation.

AMPK Activity Assay

AMPK enzyme activity was assayed in an enriched colonic mucosal homogenate[40]. Following sacrifice, proximal colons were excised from mice and opened longitudinally over ice under sterile conditions. Mucosa was removed by scraping and was homogenized in 300 μL enzyme-enrichment homogenization buffer (50 mM Tris-HCl, 250 mM mannitol, 1mM EDTA, 1mM EGTA, 50 mM NaF, 5mM $Na_4P_2O_7 \cdot 10\ H_2O$, 10% glycerol, 0.1% triton X-100, 1 mM PMSF, 1mM DTT, 1μL/mL protease inhibitor cocktail (Sigma Chemical) using a glass-stem tissue homogenizer. Subsequently, the crude homogenate was centrifuged at 15 000 g for 20 min, after which the supernatant was vortexed for 10 min following the addition of polyethylene glycol 6000 (PEG 6000, Fluka) to a final concentration of 2.5%. Samples were then centrifuged at 10 000 g for 10 min, after which the pellet was discarded and the supernatant vortexed again for 10 min following the addition of PEG 6000 to a final concentration of 6%. Samples were centrifuged at 10 000 g for 10 min, after which the supernatant was discarded while the pellet was washed once in a volume of 6% PEG 6000/homogenization buffer prior to resuspension in 50 μL enzyme-enrichment resuspension buffer (50 mM Tris-HCl, 1mM EDTA, 1mM EGTA, 50 mM NaF, 5mM $Na_4P_2O_7 \cdot 10\ H_2O$, 10% glycerol, 0.1% triton X-100, 1 mM PMSF, 1mM DTT, 1μL/mL protease inhibitor cocktail (Sigma Chemical). Protein concentrations were determined using the Bradford method and each sample diluted to a final protein concentration of 1 mg/mL in the same resuspension buffer. Prior to beginning the assay, 2 μg of the enzyme was added to each of 3 different condition assay buffers, one containing saturating amounts of AMP and the synthetic AMPK-target SAMS peptide (80 mM Hepes buffer, 160 mM NaCl, 1.6 mM EDTA, 100 mM EDTA, 200 μM SAMS peptide, 200 μM AMP, 16% glycerol, 0.1% triton X-100), and two identical to the previous buffer, excepting the addition of AMP, and AMP and SAMS peptide, respectively. All three of the assay conditions included saturating levels of ATP (1 μCi ^{32}P-γ-ATP, 200 μM unlabelled ATP, 5 mM $MgCl_2$). The assay was initiated by the addition of enzyme to the reaction tube, followed by a 5-sec vortexing, and 5 min incubation at 30°C. Following the 5 min incubation, the reaction mixture was re-vortexed and spotted on P81 Whatman filter paper (Sigma), briefly allowed to dry, and washed 3 times in 1% perchloric acid before a single wash in acetone. After sufficient time to allow the filter papers to air-dry, they were immersed in a scintillant-fluor cocktail and the activity of each sample was measured in a Beckman scintillation counter. The activity of the AMPK enzyme is reported as the difference (in pmol ATP incorporated) between the activity of the AMP-saturated sample and the sample devoid of AMP.

Brush-Border Membrane Vesicle Isolation

Animals were sacrificed by cervical dislocation and the colon removed and flushed with ice-cold PBS. The colon was opened longitudinally and the mucosa collected by scraping with a glass slide. The mucosal scraping was homogenized for 60 sec in 200 μl BBMV homogenizing buffer (300 mM mannitol, 12 mM Tris-HCl, pH 7.1) using a glass-stem homogenizer. Post-homogenization, the homogenate was diluted with 1 mL ice-cold water, before adding 12 μL 1M $MgCl_2$. The homogenate was then left to stand on ice for 40 min. Following the 40 min incubation, the homogenate was centrifuged at 8000 g for 15 min at 4°C. The supernatant was decanted, held on ice and then resuspended in 1 mL BBMV homogenization buffer by gentle repeat pipetting before adding 12 μL 1M $MgCl_2$ and left to stand on ice for 15 min. Following the 15 min incubation, the resuspended homogenate was centrifuged at 8000 g for 15 min at 4°C. Again, the supernatant was decanted, and then combined with the previously obtained supernatant, before centrifuging at 27000 g for 30 min at 4°C. The pellet was washed once in 1 mL BBMV resuspension buffer before its final resuspension in 50 μL Mono-Q buffer (50 mM β-glycerophosphate, 1 mM EGTA, 2 mM MgCl2, 0.5% Triton-X 100, pH 7.2) and subsequent analysis by western blotting.

Cell Culture Studies

T_{84} cells at passages 30-34 were grown as monolayers in a 1:1 mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F-12 medium supplemented with 15mM Na^+-HEPES buffer, pH 7.5, 14 mM $NaHCO_3$, and 5% newborn calf serum. For subculture, a cell suspension was obtained from confluent monolayers by exposing the monolayers to 0.25% trypsin and 0.9 mM EDTA in Ca^+ and Mg^+ free phosphate buffered saline. Cells were seeded at a density of 1 x 10^6 cells/1.13 cm^2 polycarbonate tissue culture treated filter and maintained at 37^0C in a 5% CO_2 atmosphere. Cultures were re-fed daily with fresh media.

To qualitatively determine whether the T_{84} cells had reached confluence, formed tight junctions, and established cell polarity, the electrical conductance and the spontaneous potential across the monolayer were determined using an EVOM voltohmeter and an STX-2 electrode set (World Precision Instruments, Sarasota, FL). To determine the effect of sustained activation of AMPK, monolayers were exposed to AICAR (1 mM) for 48 hours and then mounted in Ussing chambers for measurement of Isc, PD, and basal and forskolin-stimulated chloride fluxes.

AMPK is activated through a phosphorylation mechanism[14]. To examine the effect of epithelial exposure to oxidants on AMPK phosphorylation, monolayers were treated with H_2O_2 (1 mM, serum-free media) or $ONOO^-$ (0.1 mM, serum-free media) for 0, 5, 10, 15, 30 or 60 min prior to collection by scraping (over ice). Phosphorylation was examined by Western blotting. To examine the effect of epithelial exposure to oxidants on AMPK activity and chloride flux, monolayers were treated with H_2O_2 (2.5 mM) for 30 min. Following the 30 min incubation, the media was changed (serum free, minus oxidants). Cells were harvested by scraping over ice and AMPK activity assessed by enzymatic assay. Chloride flux was measured in Ussing chambers, and inhibition of AMPK activity was confirmed by enzymatic assay.

To determine the effect of inhibiting AMPK activity in the presence of H_2O_2, monolayers were pre-incubated with compound C at a concentration of 75 μM[39], prior to the addition of H_2O_2 (2.5 mM).

Western Blotting

T_{84} cells were harvested from 6 or 12 well plates by scraping and suspended in 0.5 ml Mono-Q buffer. Following collection, cell suspensions were sonicated on ice for 15 sec. Protein concentrations were determined using the Bradford method and samples diluted to the equivalent concentrations. Duplicate samples were separated by SDS-PAGE and either Coomassie-stained (to ensure even loading of lanes) or transferred onto PVDF membrane (Millipore). Membranes were blocked for 2 hours with 3% skim milk-TTBS (20 mM Tris, 0.5 M NaCl, 0.05% Tween 20, pH 7.4) and incubated overnight at 4°C with anti-phospho-AMPK antibody (1:1000 dilution, rabbit anti-mouse pAMPK).

Brush-border membrane vesicles prepared from animals were subjected to a single freeze-thaw cycle, and subsequently sonicated on ice for 25 sec. PVDF membranes were prepared as for T_{84} cell samples and incubated overnight at 4°C with anti-CFTR antibody (1:1000 dilution, mouse anti-human CFTR, cross-reactive to murine CFTR, C-terminus, Genzyme) for CFTR blots. Membranes were then washed 3 times with water and incubated for 2 hrs with goat anti-rabbit or goat anti-mouse secondary antibodies (Biorad; 1:3000 dilution), followed by 2 washes with TTBS. Autoradiography was performed on Kodak X-OMAT AR film using a chemiluminescence kit (Lumi-light, Amersham).

Flow Cytometry

Single cell suspensions from T_{84} monolayers were fixed with 5% formalin for 5 minutes. The fixation reaction was stopped by adding phosphate buffered saline/1% BSA. Cells were blocked with PBS/0.1% saponin/5% dried milk for 24 hr. Fixed cells were incubated with primary mAB anti-CFTR (C-terminus, Genzyme) for 1 hr followed by the secondary antibody, goat IgM:FITC (Biosource) for a second hr.

Statistical Analysis

Data are expressed as means ± SE, and statistical analyses were performed using the statistical software SigmaStat (Jandel Corp, San Rafael, CA) Differences between mean values were evaluated by analysis of variance or paired *t* test where appropriate. Specific differences were tested using Student-Newman-Keuls test.

4.3. RESULTS

Colonic Electrical Properties

Previous studies have shown that under conditions of chronic inflammation, intestinal tissue becomes hyporesponsive to secretagogues[4,6,7]. When housed under conventional conditions, IL-10 deficient mice develop a patchy chronic colitis that is limited to the colon[2]. To determine if proximal colonic segments from IL-10 deficient mice demonstrated hyporesponsiveness to secretagogues, short-circuit current (Isc) was measured in response to increasing doses of the adenylate cyclase inducing agent, forskolin. As seen in Figure 4.1, colons from IL-10 deficient mice had a significantly reduced Isc response to increasing doses of forskolin, compared with responses seen in wild-type mice. The measurement of unidirectional chloride fluxes confirmed the increase in serosal-to-mucosal movement of chloride in colons from wild-type mice, and the absence of such a response in IL-10 deficient mice (Figure 4.2).

As expected, the delta Isc response to forskolin in wild-type mice was largely associated with Cl^- and HCO_3^-[41,42]. Removal of chloride resulted in a ~55% reduction ($p<0.05$; Figure 4.3) in forskolin-stimulated Isc, and simultaneous removal of both Cl^- and HCO_3^- resulted in virtually no Isc response. These findings are in agreement with other studies showing that the rodent colon is dominated by electroneutral absorption of sodium; thus the Isc measured is primarily due to chloride and bicarbonate movement[41,42].

Basal electrical parameters in the colon were similar between wild-type (PD: 1.6 ± 0.3 mV; Isc 28.0 ± 3.7 $\mu A/cm^2$) and IL-10 deficient mice (PD: 1.2 +/- 0.3 mV; Isc: 21.6 +/- 2.5 $\mu A/cm^2$). Basal conductance (G) was also similar, and did not change significantly in response to forskolin in either wild-type (17.6 +/- 1.1 vs 22.7 +/- 3.3 mS/cm^2) or IL-10 deficient mice (22.9 +/- 3.7 vs 18.9 +/- 2.2 mS/cm^2) indicating that the changes in chloride serosal-to-mucosal fluxes were not due to increased paracellular movement of chloride.

Under inflammatory conditions, the levels of prostaglandins would be expected to be increased[43]; thus, in the IL-10 deficient mice, the failure to respond to forskolin may have been related to already elevated intracellular cAMP levels. To determine if the secretory defect seen in the IL-10 deficient mice was due to elevated cAMP levels, colonic tissue was incubated with indomethacin to reduce prostanoid activity, and then stimulated with forskolin. As seen in Table 4.1, the presence of indomethacin did not alter the hyporesponsiveness seen in the IL-10 deficient mice. These data would suggest that the secretory defect in the proximal colon of IL-10 deficient mice was not due to elevated epithelial cAMP levels.

CFTR Brush-Border Membrane Localization

In the colon, both calcium and cAMP-mediated chloride secretion are primarily mediated by CFTR[35]. Previous studies have suggested that activation of CFTR by cAMP can involve an increased number of transporters being inserted in the brush border membrane, either by increasing the rate of exocytosis or decreasing the rate of endocytosis of CFTR-containing membrane vesicles[44-46]. Examination of isolated brush border vesicles from wild-type and IL-10 deficient mice by western blotting techniques demonstrated a reduction in CFTR expression associated with the brush-border membrane in IL-10 deficient mice (Figure 4.4). This would suggest that the inability of IL-10 deficient mice to respond to forskolin was associated with reductions of CFTR associated with the brush-border membrane fraction.

AMPK Activity Assay

AMPK is activated in response to metabolic stress and acts to phosphorylate and inhibit several biosynthetic enzymes, thereby preserving cellular ATP levels during periods of metabolic depletion[17]. We have previously shown that chronic inflammation in the IL-10 deficient mouse is associated with high levels of peroxynitrite suggesting enhanced nitrosative stress[47]. Thus, to determine if AMPK activity was up-regulated in inflamed colons in IL-10 deficient mice, colonic tissue was examined in IL-10 deficient mice at 2 weeks of age (in the absence of any inflammation) and at 6 weeks of age (after inflammation develops) and compared with age-matched controls. At 2 weeks of age, there was no significant difference in the activity of the enzyme between the two groups; however, by 6 weeks of age, a significant increase in enzymatic activity was evident in the IL-10 deficient group (Figure 4.5). This data would suggest that the activity of AMPK was upregulated in IL-10 deficient mice under conditions of colonic inflammation.

Effect of AMPK Inhibition on Colonic Chloride Secretion

Recent studies have shown that AMP-activated protein kinase (AMPK) interacts with CFTR and inhibits its activity[36,37]. Having shown that colonic tissue from IL-10 deficient mice demonstrated enhanced levels of AMPK activity and also was unresponsive to forskolin stimulation, we carried out a series of experiments to determine if inhibition of AMPK activity would restore cAMP-mediated chloride secretion. Colons from IL-10 deficient mice were treated *in vitro* with compound C and chloride secretion assessed. Compound C is a potent reversible inhibitor of AMPK that is competitive with ATP and which does not exhibit significant inhibition of other kinases structurally related to AMPK[39]. As seen in Figure 4.6, colonic tissue from IL-10 deficient mice did not respond to forskolin with an increase in serosal-to-mucosal chloride flux. In contrast, colonic tissue from IL-10 deficient mice pre-treated with compound C demonstrated a complete restoration in serosal-to-mucosal chloride flux to levels seen in wild-type mice. There was no significant effect of forskolin on conductance in either colons from IL-10 deficient mice (26.4 +/- 2.4 vs 30.1 +/- 2.9 mS/cm^2) or in colons treated with compound C (21.3 +/- 2.8 vs 23.3 +/- 2.2 mS/cm^2).

Effect of AMPK Activation on Colonic Chloride Secretion

To further correlate the deficiency in chloride secretion seen in the IL-10 deficient mouse with AMPK activity, healthy colonic tissue from wild-type mice was treated *in vitro* with AICAR and chloride flux measured (Figure 4.6). Incubation with the AMPK activator AICAR results in accumulation of the monophosphorylated derivative, 5-aminoimidazole-4-carboxamide 1-beta-dribofuranotide (ZMP), the active intracellular form of AICAR, within the cell[48]. ZMP mimics both activating effects of AMP on AMPK; that is, a direct allosteric activation, and promotion of phosphorylation by AMPKK[28]. While activating AMPK, AICAR does not perturb the cellular concentration of ATP, nor its metabolites ADP or AMP[28]. As seen in Figure 4.6, AICAR-treated colonic tissue from normal healthy mice functionally resembled tissue from IL-10 deficient mice, in that the tissue did not respond to forskolin. There was no significant effect of forskolin on conductance in either colons from wild-type mice (27.2 +/- 1.6 vs 31.8 +/- 2.5 mS/cm^2) or in colons treated with AICAR (22.9 +/- 1.8 vs 23.8 +/- 2.3 mS/cm^2). This data supports the hypothesis that activation of AMPK in colonic epithelial tissue occurs under conditions of chronic inflammation and that this activation is linked with decreases in cAMP-mediated chloride secretion.

Cell Culture Experiments

Treatment of T_{84} Monolayers with H_2O_2

To further link metabolic stress, AMPK activity, and CFTR down-regulation, we carried out a series of experiments in T_{84} epithelial monolayers using either H_2O_2 or $ONOO^-$ to induce metabolic stress. AMPK is activated by a phosphorylation mechanism[12]. Thus, initial experiments were carried out exposing monolayers to either H_2O_2 or $ONOO^-$ and assessing AMPK phosphorylation. Both H_2O_2 and $ONOO^-$ exposure resulted in a time-dependent increase in phosphorylation (Figure 4.7), indicating that T_{84} monolayers respond to oxidants with increased AMPK activity. A second series of experiments was then carried out to link H_2O_2 exposure with chloride flux. As seen in Figure 4.8.A, exposure of cells to H_2O_2 resulted in enhanced AMPK activity as measured by enzymatic activity, and a reduction in forskolin-stimulated serosal-to-mucosal flux of chloride (Figure 4.8.B), as measured in Ussing chambers. To confirm the role of AMPK in the reduction of forskolin-stimulated chloride flux, monolayers were pre-treated with compound C prior to H_2O_2 exposure, and chloride flux measured. The inhibition of AMPK activity by compound C was confirmed by enzymatic assay (Figure 4.8.A). As seen in Figure 4.8.B, inhibition of AMPK activity prevented the H_2O_2 –induced reduction in forskolin stimulated chloride flux. These data strongly suggest that the secretory hyporesponsiveness seen in epithelial tissue in the presence of oxidants is related to the activation of AMPK.

Treatment of T_{84} Monolayers with AICAR

Under conditions of chronic inflammation, AMPK activity may be elevated for significant periods of time. To examine the effects of chronic activation of AMPK on epithelial function, T_{84} epithelial monolayers were incubated with AICAR for 48 hr, and monolayer resistance, short-circuit current, and chloride fluxes assessed. The presence of AICAR significantly increased conductance, while reducing Isc, PD, and Isc response to forskolin (Table 4.2). In addition, serosal-to-mucosal flux of chloride in response to forskolin was significantly reduced in the presence of AICAR (Table 4.2). This was associated with decreased levels of CFTR in the apical membrane as measured by flow cytometry (Figure 4.9). These data suggest that long-term activation of AMPK has significant consequences on several aspects of ionic epithelial function.

4.4. DISCUSSION

In the present study, we have demonstrated that AMPK activity is up-regulated under conditions of chronic inflammation in colons of IL-10 deficient mice; furthermore, this up-regulation was associated with an inability of colonic tissue to respond to cAMP-mediated chloride secretion. Pharmacological inhibition of AMPK resulted in a restoration of chloride secretion in IL-10 deficient mice; conversely, stimulation of AMPK activity in wild-type mice resulted in an inhibition of forskolin-stimulated chloride secretion. *In vitro* studies in cultured epithelial cells demonstrated that an H_2O_2-induced activation of AMPK resulted in a down-regulation of CFTR membrane expression coupled with a decrease in chloride secretion in response to secretagogues. This study is the first to describe a potential role for AMPK in mediating the secretory abnormalities seen under conditions of chronic inflammation.

Diarrhea is commonly seen clinically in patients with inflammatory bowel disease[49]. Although it was initially suggested that inflammation associated diarrhea occurred as a result of inflammatory mediators acting as secretagogues and stimulating chloride secretion[50], recent evidence appears to refute this concept[5]. Indeed, the fluid accumulation and diarrhea seen in patients with inflammatory bowel disease may occur as a result of decreased Na^+ absorption and Cl^- secretory processes rather than a stimulation of ion secretion[7,51,52]. Data from this study supports this concept and further implicates activation of AMPK as the intracellular mediator responsible for these transport abnormalities.

In the IL-10 deficient mouse, colonic inflammation is associated with increased secretion of pro-inflammatory cytokines and high levels of nitrosative stress[47]. Previous studies in animal models of colitis[4,31,53] and cell culture models[51,54,55] have shown that exposure of epithelial cells to either nitrogen- or oxygen-derived species or pro-inflammatory cytokines such as IFNγ and TNFα results in the induction of a hypo-responsive chloride secretory state. In our study, while basal chloride secretion was similar between wild-type mice and IL-10 deficient mice, adult IL-10 deficient mice with established colitis exhibited an inability to respond to forskolin with an up-regulation of colonic chloride secretion. Previous studies have shown a similar pattern, with basal electrogenic ion transport remaining unchanged in a rat model of colitis, while the tissue response to a phosphodiesterase inhibitor IBMX, was significantly reduced[56]. Inflamed human intestinal tissue has also shown a similar lack of response to theophylline as compared with control tissue[9].

Chloride secretion through CFTR is a major determinant of mucosal hydration throughout the intestinal tract[53,57]. CFTR plays an important role in colonic absorption and secretion of salt and water and appears to be critical for determining the overall rate of transepithelial ion transport[35]. CFTR is activated in epithelial cells through hormone and neurotransmitter-induced cAMP-mediated signaling[35]. Thus, in that forskolin acts to stimulate chloride secretion through activation of the adenylate cyclase pathway and a subsequent rise in intracellular cAMP levels, the failure of tissue to respond to forskolin-stimulation could be attributed to already elevated cAMP levels in colonocytes. This may occur as a consequence of increased levels of inflammatory mediators in the mucosa, including leukotriene B_4, prostaglandin E_2, interleukin-1, and reactive oxygen metabolites[43] – all of which have been shown to increase cAMP levels[57]. However, in our study, pre-treatment of colonic tissue with indomethacin to inhibit prostanoid activity had no effect on the hypo-responsiveness of tissue to forskolin, arguing against already elevated intracellular cAMP as being the mechanism behind the failure of chronically inflamed tissue to respond to forskolin. In addition, previous studies have shown enterocyte cAMP levels to be decreased under chronic inflammatory conditions, as opposed to increased, as occurs under acute inflammatory conditions[6].

CFTR belongs to the family of ATP-binding cassette proteins and requires ATP binding and hydrolysis for activity[58]. It has been proposed that the ATP requirement for CFTR activity allows for the coupling of CFTR with cellular energetics[11]. Recently, Hallows et al have shown that AMPK and CFTR co-localize in epithelial cells, and AMPK phosphorylation of CFTR *in vitro* inhibits cAMP-activated CFTR conductance in Xenopus oocytes[59] and T_{84} cells[36]. Our data, both in whole animals and in cell culture, supports the concept of AMPK being an endogenous inhibitor of CFTR activity. AMPK was up-regulated in colons from IL-10 deficient mice only under inflammatory conditions, thus the up-regulation was not due to the lack of IL-10 *per se*; and furthermore, pharmacological inhibition of AMPK activity resulted in restoration of tissue responsiveness to forskolin. Finally, in wild-type mice, stimulation of AMPK with AICAR produced a profound inhibition of CFTR activity.

The mechanism(s) underlying AMPK regulation of CFTR channel activity under chronic inflammatory conditions may involve either a down-regulation of CFTR protein expression, an inhibition of protein maturation or increased degradation[60], or an inhibition of CFTR protein insertion into the brush border membrane during stimulus of secretion[61,62]. In addition, CFTR activity can be regulated through a phosphorylation mechanism involving cAMP and protein kinase A (PKA)[63,64]. Activation of PKA by rises in intracellular cAMP results in the phosphorylation of the cytoplasmic domain of CFTR and a resultant activation of gating by a destabilization of channel closed states[65]. Indeed, the regulation of CFTR channel activity and/or apical membrane expression is complex and occurs at several different levels. Numerous proteins have been shown to interact with CFTR, including syntaxin 1A[66], the PDZ domain-containing proteins NHERF and CAP70[67], and the m2-subunit of the AP-2 adaptor protein complex[68]. The catalytic subunit of AMPK has also been shown to bind to a region near the COOH terminus of CFTR and result in an AMPK-dependent phosphorylation *in vitro*[59]. Thus, although PKA-mediated phosphorylation is the best-characterized mechanism for modifying CFTR activity, AMPK phosphorylation may have an equally important role in regulating chloride secretion. Supporting this concept are recent studies in human lung epithelial cells, which demonstrated that pharmacologic activation of AMPK resulted in an inhibition of single-channel gating, rather than a reduction of CFTR expression in the apical membrane[37]. However, whether this occurs by a direct phosphorylation of CFTR by AMPK remains to be shown, as AMPK may also induce its effects through modifying the activity of PKA or other kinases/ phosphatases involved in the regulation of CFTR expression or activity. Indeed, AMPK phosphorylates and inhibits numerous rate-limiting biosynthetic enzymes in order to maintain cellular ATP stores during metabolic stress[11].

In this study, the levels of CFTR protein appear to be reduced in IL-10 deficient mice, although whether this involves only a reduction of CFTR channels in the apical membrane or also a reduction in intracellular protein remains to be clarified. The use of brush border vesicles from mice colon cannot differentiate between CFTR in certain endosomal compartments and CFTR expressed in brush border membranes. Thus, the reduction observed in brush border membranes from IL-10 deficient mice may also have been due to an overall reduction of CFTR within the cell. However, in that basal levels of chloride secretion were similar in wild-type and IL-10 deficient mice, and, further, inhibition of AMPK with compound C resulted in the rapid restoration of chloride secretion, it would appear that the amount of CFTR that was present in colonocytes in IL-10 deficient mice was sufficient to manifest chloride secretion once the inhibitory effect of AMPK was removed. Whether this involved increased insertion of CFTR into the brush border membrane, or an alteration in the phosphorylation state of CFTR remains to be clarified. Conflicting results have been obtained regarding the effect of inflammation on CFTR expression. Indeed, both down-regulation and no change in expression has been reported[6,54,69]. These differences may be related to either the model system studied, or the length of time of colitis. Further experiments will be necessary to resolve this issue.

In conclusion, the role of AMPK in mediating the chloride transport abnormalities seen under conditions of chronic inflammation would appear to be one of suppression. This inhibition of chloride secretion by AMPK would serve to reduce CFTR-dependent ATP utilization, in order to preserve intracellular levels of ATP. Furthermore, such an association between AMPK and CFTR would allow for the efficient coupling of intracellular metabolism with the maintenance of transcellular epithelial ion transport.

4.5. REFERENCES

1. Tuzun, A., Erdil, A., Inal, V., Aydin, A., Bagci, S., Yesilova, Z., Sayal, A., Karaeren, N. & Dagalp, K. Oxidative stress and antioxidant capacity in patients with inflammatory bowel disease. *Clin Biochem* **35**, 569-72 (2002).
2. Berg, D.J., Davidson, N., Kuhn, R., Muller, W., Menon, S., Holland, G., Thompson-Snipes, L., Leach, M.W. & Rennick, D. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest* **98**, 1010-20 (1996).
3. Berg, S., Sappington, P.L., Guzik, L.J., Delude, R.L. & Fink, M.P. Proinflammatory cytokines increase the rate of glycolysis and adenosine-5'-triphosphate turnover in cultured rat enterocytes. *Crit Care Med* **31**, 1203-12 (2003).
4. Bell, C.J., Gall, D.G. & Wallace, J.L. Disruption of colonic electrolyte transport in experimental colitis. *Am J Physiol* **268**, G622-30 (1995).
5. Musch, M.W., Clarke, L.L., Mamah, D., Gawenis, L.R., Zhang, Z., Ellsworth, W., Shalowitz, D., Mittal, N., Efthimiou, P., Alnadjim, Z., Hurst, S.D., Chang, E.B. & Barrett, T.A. T cell activation causes diarrhea by increasing intestinal permeability and inhibiting epithelial Na+/K+-ATPase. *J Clin Invest* **110**, 1739-47 (2002).
6. Sanchez de Medina, F., Perez, R., Martinez-Augustin, O., Gonzalez, R., Lorente, M.D., Galvez, J. & Zarzuelo, A. Disturbances of colonic ion secretion in inflammation: role of the enteric nervous system and cAMP. *Pflugers Arch* **444**, 378-88 (2002).
7. Sundaram, U. & West, A.B. Effect of chronic inflammation on electrolyte transport in rabbit ileal villus and crypt cells. *Am J Physiol* **272**, G732-41 (1997).
8. Greig, E. & Sandle, G.I. Diarrhea in ulcerative colitis. The role of altered colonic sodium transport. *Ann N Y Acad Sci* **915**, 327-32 (2000).
9. Hubel, K.A. & Renquist, K.S. Ion transport in normal and inflamed human jejunum in vitro. Changes with electric field stimulation and theophylline. *Dig Dis Sci* **35**, 815-20 (1990).
10. Lohi, H., Makela, S., Pulkkinen, K., Hoglund, P., Karjalainen-Lindsberg, M.L., Puolakkainen, P. & Kere, J. Upregulation of CFTR expression but not SLC26A3 and SLC9A3 in ulcerative colitis. *Am J Physiol Gastrointest Liver Physiol* **283**, G567-75 (2002).
11. Hardie, D.G. & Carling, D. The AMP-activated protein kinase--fuel gauge of the mammalian cell? *Eur J Biochem* **246**, 259-73 (1997).
12. Hardie, D.G. & Hawley, S.A. AMP-activated protein kinase: the energy charge hypothesis revisited. *Bioessays* **23**, 1112-9 (2001).
13. Carling, D., Clarke, P.R., Zammit, V.A. & Hardie, D.G. Purification and characterization of the AMP-activated protein kinase. Copurification of acetyl-CoA carboxylase kinase and 3-hydroxy-3-methylglutaryl-CoA reductase kinase activities. *Eur J Biochem* **186**, 129-36 (1989).

14. Cheung, P.C., Salt, I.P., Davies, S.P., Hardie, D.G. & Carling, D. Characterization of AMP-activated protein kinase gamma-subunit isoforms and their role in AMP binding. *Biochem J* **346 Pt 3**, 659-69 (2000).
15. Hardie, D.G., Salt, I.P., Hawley, S.A. & Davies, S.P. AMP-activated protein kinase: an ultrasensitive system for monitoring cellular energy charge. *Biochem J* **338 (Pt 3)**, 717-22 (1999).
16. Kemp, B.E., Mitchelhill, K.I., Stapleton, D., Michell, B.J., Chen, Z.P. & Witters, L.A. Dealing with energy demand: the AMP-activated protein kinase. *Trends Biochem Sci* **24**, 22-5 (1999).
17. Corton, J.M., Gillespie, J.G. & Hardie, D.G. Role of the AMP-activated protein kinase in the cellular stress response. *Curr Biol* **4**, 315-24 (1994).
18. Hayashi, T., Hirshman, M.F., Fujii, N., Habinowski, S.A., Witters, L.A. & Goodyear, L.J. Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. *Diabetes* **49**, 527-31 (2000).
19. Musi, N., Hayashi, T., Fujii, N., Hirshman, M.F., Witters, L.A. & Goodyear, L.J. AMP-activated protein kinase activity and glucose uptake in rat skeletal muscle. *Am J Physiol Endocrinol Metab* **280**, E677-84 (2001).
20. Salt, I.P., Johnson, G., Ashcroft, S.J. & Hardie, D.G. AMP-activated protein kinase is activated by low glucose in cell lines derived from pancreatic beta cells, and may regulate insulin release. *Biochem J* **335 (Pt 3)**, 533-9 (1998).
21. Marsin, A.S., Bouzin, C., Bertrand, L. & Hue, L. The stimulation of glycolysis by hypoxia in activated monocytes is mediated by AMP-activated protein kinase and inducible 6-phosphofructo-2-kinase. *J Biol Chem* **277**, 30778-83 (2002).
22. Taylor, C.T., Lisco, S.J., Awtrey, C.S. & Colgan, S.P. Hypoxia inhibits cyclic nucleotide-stimulated epithelial ion transport: role for nucleotide cyclases as oxygen sensors. *J Pharmacol Exp Ther* **284**, 568-75 (1998).
23. Unno, N., Baba, S. & Fink, M.P. Cytosolic ionized Ca2+ modulates chemical hypoxia-induced hyperpermeability in intestinal epithelial monolayers. *Am J Physiol* **274**, G700-8 (1998).
24. Hardie, D.G., Carling, D. & Carlson, M. The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu Rev Biochem* **67**, 821-55 (1998).
25. Hue, L., Beauloye, C., Marsin, A.S., Bertrand, L., Horman, S. & Rider, M.H. Insulin and ischemia stimulate glycolysis by acting on the same targets through different and opposing signaling pathways. *J Mol Cell Cardiol* **34**, 1091-7 (2002).
26. DuVall, M.D., Guo, Y. & Matalon, S. Hydrogen peroxide inhibits cAMP-induced Cl- secretion across colonic epithelial cells. *Am J Physiol* **275**, C1313-22 (1998).
27. Hyslop, P.A., Hinshaw, D.B., Halsey, W.A., Jr., Schraufstatter, I.U., Sauerheber, R.D., Spragg, R.G., Jackson, J.H. & Cochrane, C.G. Mechanisms of oxidant-mediated cell injury. The glycolytic and mitochondrial pathways of ADP phosphorylation are major intracellular

targets inactivated by hydrogen peroxide. *J Biol Chem* **263**, 1665-75 (1988).

28. Stein, S.C., Woods, A., Jones, N.A., Davison, M.D. & Carling, D. The regulation of AMP-activated protein kinase by phosphorylation. *Biochem J* **345 Pt 3**, 437-43 (2000).
29. Dyck, J.R., Kudo, N., Barr, A.J., Davies, S.P., Hardie, D.G. & Lopaschuk, G.D. Phosphorylation control of cardiac acetyl-CoA carboxylase by cAMP-dependent protein kinase and 5'-AMP activated protein kinase. *Eur J Biochem* **262**, 184-90 (1999).
30. Foretz, M., Carling, D., Guichard, C., Ferre, P. & Foufelle, F. AMP-activated protein kinase inhibits the glucose-activated expression of fatty acid synthase gene in rat hepatocytes. *J Biol Chem* **273**, 14767-71 (1998).
31. Fryer, L.G., Hajduch, E., Rencurel, F., Salt, I.P., Hundal, H.S., Hardie, D.G. & Carling, D. Activation of glucose transport by AMP-activated protein kinase via stimulation of nitric oxide synthase. *Diabetes* **49**, 1978-85 (2000).
32. Russell, R.R., 3rd, Bergeron, R., Shulman, G.I. & Young, L.H. Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. *Am J Physiol* **277**, H643-9 (1999).
33. Hardie, D.G. & Pan, D.A. Regulation of fatty acid synthesis and oxidation by the AMP-activated protein kinase. *Biochem Soc Trans* **30**, 1064-70 (2002).
34. Leclerc, I., Viollet, B., da Silva Xavier, G., Kahn, A. & Rutter, G.A. Role of AMP-activated protein kinase in the regulation of gene transcription. *Biochem Soc Trans* **30**, 307-11 (2002).
35. Greger, R. Role of CFTR in the colon. *Annu Rev Physiol* **62**, 467-91 (2000).
36. Hallows, K.R., Kobinger, G.P., Wilson, J.M., Witters, L.A. & Foskett, J.K. Physiological modulation of CFTR activity by AMP-activated protein kinase in polarized T84 cells. *Am J Physiol Cell Physiol* **284**, C1297-308 (2003).
37. Hallows, K.R., McCane, J.E., Kemp, B.E., Witters, L.A. & Foskett, J.K. Regulation of channel gating by AMP-activated protein kinase modulates cystic fibrosis transmembrane conductance regulator activity in lung submucosal cells. *J Biol Chem* **278**, 998-1004 (2003).
38. Asfaha, S., Bell, C.J., Wallace, J.L. & MacNaughton, W.K. Prolonged colonic epithelial hyporesponsiveness after colitis: role of inducible nitric oxide synthase. *Am J Physiol* **276**, G703-10 (1999).
39. Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M.F., Goodyear, L.J. & Moller, D.E. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* **108**, 1167-74 (2001).
40. Davies, S.P., Carling, D. & Hardie, D.G. Tissue distribution of the AMP-activated protein kinase, and lack of activation by cyclic-AMP-dependent protein kinase, studied using a specific and sensitive peptide assay. *Eur J Biochem* **186**, 123-8 (1989).

41. Binder, H.J., Foster, E.S., Budinger, M.E. & Hayslett, J.P. Mechanism of electroneutral sodium chloride absorption in distal colon of the rat. *Gastroenterology* **93**, 449-55 (1987).
42. Rajendran, V.M. & Binder, H.J. Characterization and molecular localization of anion transporters in colonic epithelial cells. *Ann N Y Acad Sci* **915**, 15-29 (2000).
43. Wallace, J.L. Prostaglandin biology in inflammatory bowel disease. *Gastroenterol Clin North Am* **30**, 971-80 (2001).
44. Cunningham, S.A., Frizzell, R.A. & Morris, A.P. Vesicular targeting and the control of ion secretion in epithelial cells: implications for cystic fibrosis. *J Physiol* **482**, 27S-30S (1995).
45. Morris, A.P., Cunningham, S.A. & Frizzell, R.A. CFTR targeting in epithelial cells. *J Bioenerg Biomembr* **25**, 21-6 (1993).
46. Takahashi, A., Watkins, S.C., Howard, M. & Frizzell, R.A. CFTR-dependent membrane insertion is linked to stimulation of the CFTR chloride conductance. *Am J Physiol* **271**, C1887-94 (1996).
47. Jijon, H.B., Churchill, T., Malfair, D., Wessler, A., Jewell, L.D., Parsons, H.G. & Madsen, K.L. Inhibition of poly(ADP-ribose) polymerase attenuates inflammation in a model of chronic colitis. *Am J Physiol Gastrointest Liver Physiol* **279**, G641-51 (2000).
48. Corton, J.M., Gillespie, J.G., Hawley, S.A. & Hardie, D.G. 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? *Eur J Biochem* **229**, 558-65 (1995).
49. Burgel, N., Bojarski, C., Mankertz, J., Zeitz, M., Fromm, M. & Schulzke, J.D. Mechanisms of diarrhea in collagenous colitis. *Gastroenterology* **123**, 433-43 (2002).
50. Ciancio, M.J. & Chang, E.B. Epithelial secretory response to inflammation. *Ann N Y Acad Sci* **664**, 210-21 (1992).
51. Sugi, K., Musch, M.W., Field, M. & Chang, E.B. Inhibition of Na+,K+-ATPase by interferon gamma down-regulates intestinal epithelial transport and barrier function. *Gastroenterology* **120**, 1393-403 (2001).
52. Sundaram, U., Wisel, S., Rajendren, V.M. & West, A.B. Mechanism of inhibition of Na+-glucose cotransport in the chronically inflamed rabbit ileum. *Am J Physiol* **273**, G913-9 (1997).
53. Kunzelmann, K. & Mall, M. Electrolyte transport in the mammalian colon: mechanisms and implications for disease. *Physiol Rev* **82**, 245-89 (2002).
54. Colgan, S.P., Parkos, C.A., Matthews, J.B., D'Andrea, L., Awtrey, C.S., Lichtman, A.H., Delp-Archer, C. & Madara, J.L. Interferon-gamma induces a cell surface phenotype switch on T84 intestinal epithelial cells. *Am J Physiol* **267**, C402-10 (1994).
55. Fish, S.M., Proujansky, R. & Reenstra, W.W. Synergistic effects of interferon gamma and tumour necrosis factor alpha on T84 cell function. *Gut* **45**, 191-8 (1999).
56. Kachur, J.F., Keshavarzian, A., Sundaresan, R., Doria, M., Walsh, R., de las Alas, M.M. & Gaginella, T.S. Colitis reduces short-circuit current

response to inflammatory mediators in rat colonic mucosa. *Inflammation* **19**, 245-59 (1995).

57. Barrett, K.E. & Keely, S.J. Chloride secretion by the intestinal epithelium: molecular basis and regulatory aspects. *Annu Rev Physiol* **62**, 535-72 (2000).
58. Riordan, J.R., Rommens, J.M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.L. & et al. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* **245**, 1066-73 (1989).
59. Hallows, K.R., Raghuram, V., Kemp, B.E., Witters, L.A. & Foskett, J.K. Inhibition of cystic fibrosis transmembrane conductance regulator by novel interaction with the metabolic sensor AMP-activated protein kinase. *J Clin Invest* **105**, 1711-21 (2000).
60. Bebok, Z., Varga, K., Hicks, J.K., Venglarik, C.J., Kovacs, T., Chen, L., Hardiman, K.M., Collawn, J.F., Sorscher, E.J. & Matalon, S. Reactive oxygen nitrogen species decrease cystic fibrosis transmembrane conductance regulator expression and cAMP-mediated Cl- secretion in airway epithelia. *J Biol Chem* **277**, 43041-9 (2002).
61. Ameen, N.A., van Donselaar, E., Posthuma, G., de Jonge, H., McLaughlin, G., Geuze, H.J., Marino, C. & Peters, P.J. Subcellular distribution of CFTR in rat intestine supports a physiologic role for CFTR regulation by vesicle traffic. *Histochem Cell Biol* **114**, 219-28 (2000).
62. Howard, M., Jiang, X., Stolz, D.B., Hill, W.G., Johnson, J.A., Watkins, S.C., Frizzell, R.A., Bruton, C.M., Robbins, P.D. & Weisz, O.A. Forskolin-induced apical membrane insertion of virally expressed, epitope-tagged CFTR in polarized MDCK cells. *Am J Physiol Cell Physiol* **279**, C375-82 (2000).
63. Stutts, M.J., Canessa, C.M., Olsen, J.C., Hamrick, M., Cohn, J.A., Rossier, B.C. & Boucher, R.C. CFTR as a cAMP-dependent regulator of sodium channels. *Science* **269**, 847-50 (1995).
64. Tousson, A., Fuller, C.M. & Benos, D.J. Apical recruitment of CFTR in T-84 cells is dependent on cAMP and microtubules but not Ca2+ or microfilaments. *J Cell Sci* **109 (Pt 6)**, 1325-34 (1996).
65. Winter, M.C. & Welsh, M.J. Stimulation of CFTR activity by its phosphorylated R domain. *Nature* **389**, 294-6 (1997).
66. Cormet-Boyaka, E., Di, A., Chang, S.Y., Naren, A.P., Tousson, A., Nelson, D.J. & Kirk, K.L. CFTR chloride channels are regulated by a SNAP-23/syntaxin 1A complex. *Proc Natl Acad Sci U S A* **99**, 12477-82 (2002).
67. Raghuram, V., Mak, D.D. & Foskett, J.K. Regulation of cystic fibrosis transmembrane conductance regulator single-channel gating by bivalent PDZ-domain-mediated interaction. *Proc Natl Acad Sci U S A* **98**, 1300-5 (2001).
68. Weixel, K.M. & Bradbury, N.A. Mu 2 binding directs the cystic fibrosis transmembrane conductance regulator to the clathrin-mediated endocytic pathway. *J Biol Chem* **276**, 46251-9 (2001).

69. Besancon, F., Przewlocki, G., Baro, I., Hongre, A.S., Escande, D. & Edelman, A. Interferon-gamma downregulates CFTR gene expression in epithelial cells. *Am J Physiol* **267**, C1398-404 (1994).

4.6. EXPERIMENTAL RESULTS

Figure 4.1. Colonic short-circuit response to increasing concentrations of forskolin (10^{-8}-10^{-4} M) in wild-type and IL-10 deficient mice

Colonic tissue from wild-type mice (n=4) exhibited significantly greater Isc response to forskolin compared with colons from IL-10 deficient mice (n=4). Values are means ± SE.
*$p<0.05$ control compared with wild-type mice.

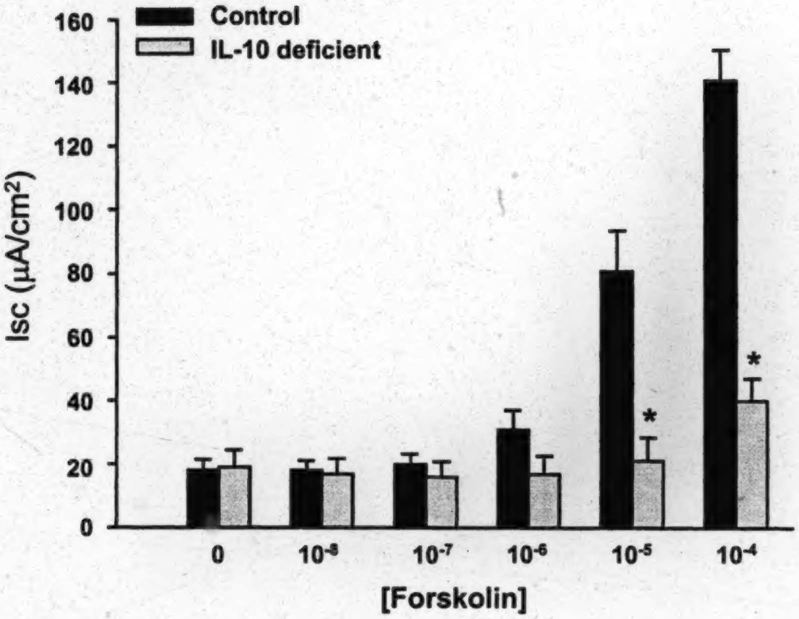


Figure 4.2. Basal and forskolin-stimulated chloride fluxes in colons from wild-type and IL-10 deficient mice

There were no differences in basal mucosal-to-serosal (M-S) or serosal-to-mucosal (S-M) chloride fluxes between the 2 groups. Colons from wild-type mice responded to forskolin (10^{-4} M) with a significant increase in serosal-to-mucosal movement of chloride. In contrast, colons from IL-10 deficient mice did not respond to forskolin. Values are means ± SE.
Wild-type animals n=4; IL-10 deficient animals n=6.
*p<0.05 forskolin compared with basal period.

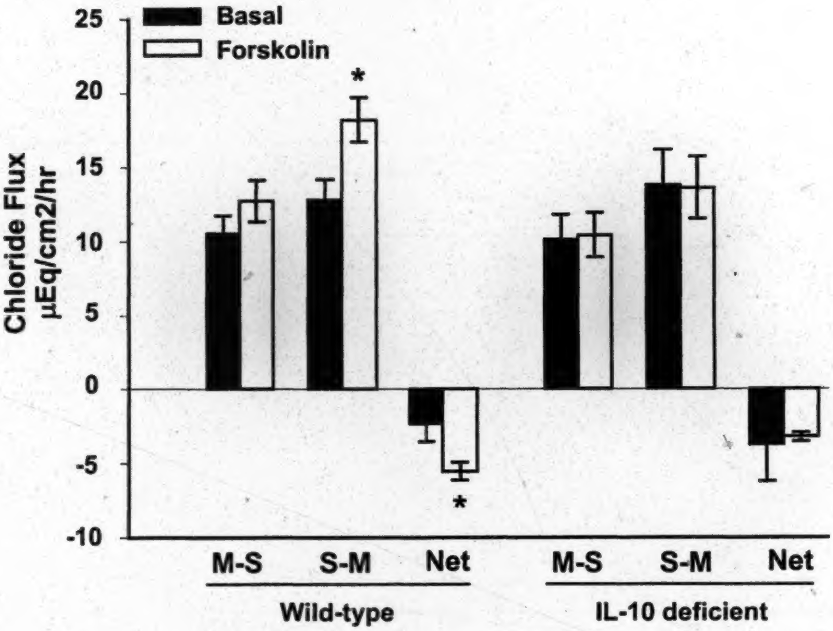


Figure 4.3. Anion dependency of the colonic Isc response to forskolin (10^{-4} M) in wild-type and IL-10 deficient mice

Colons from wild-type mice responded to forskolin with a large increase in Isc. Removal of chloride resulted in a decreased response, whereas removal of bicarbonate eliminated the response. IL-10 deficient mice did not respond to forskolin with any significant increase in Isc. Values are means ± SE of 4 animals in each group.

*$p<0.01$ compared with wild-type mice; $^{\#}p<0.01$ compared with Ringers solution-exposed mice; $^{+}p<0.05$ compared with chloride-free control mice.

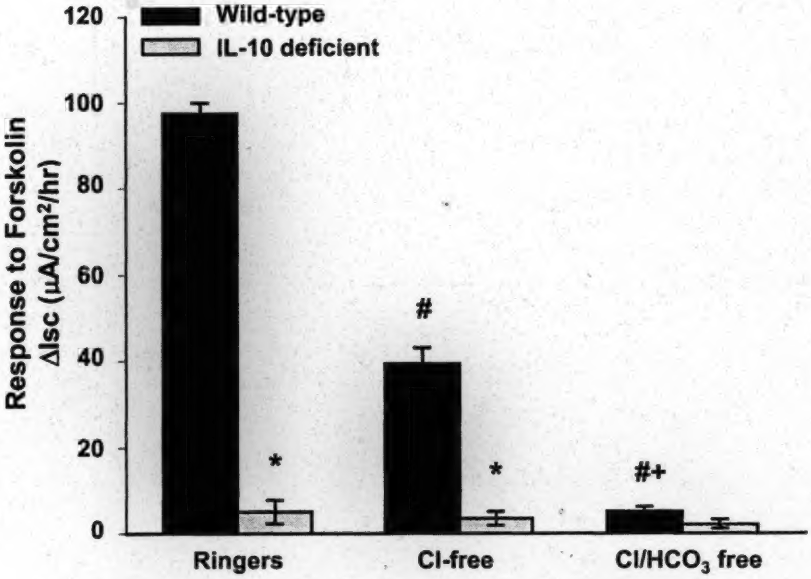


Figure 4.4. Representative Western blot of cystic fibrosis transmembrane conductance regulator (CFTR) expression in colonic mucosa brush-border vesicles prepared from wild-type and IL-10 deficient mice

Wild-type mice demonstrated higher levels of CFTR expression compared with IL-10 deficient mice. Each lane represents pooled brush-border membrane isolates from 2 animals. The experiment was repeated 2 times.

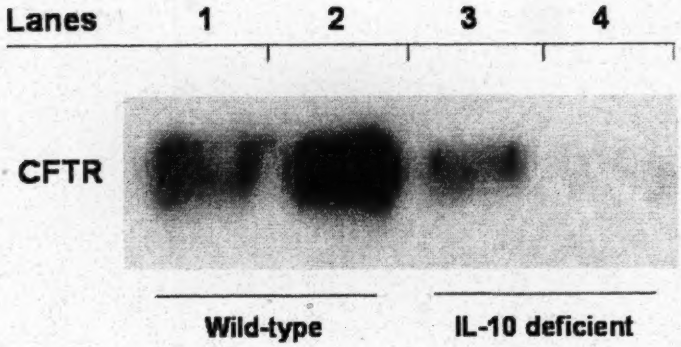


Figure 4.5. AMP-activated protein kinase (AMPK) activity in colonic mucosa of 2 and 6 week old wild-type and IL-10 deficient mice

There was no difference in AMPK activity at 2 wk of age between the 2 groups. Following the development of inflammation in IL-10 deficient mice, the activity of AMPK increased. Values are means ± SE; n = 6–8 mice at each time point.
*p<0.01 compared with wild-type mice.

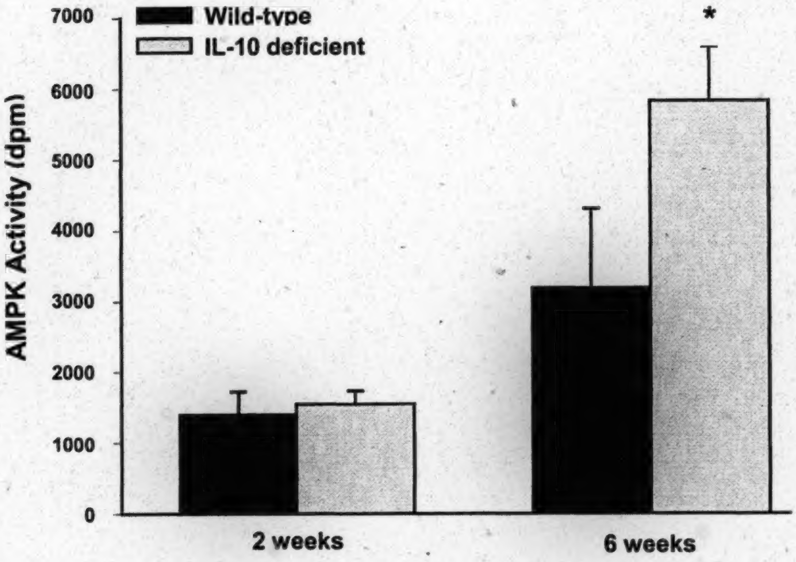


Figure 4.6. Change in chloride flux in colons from wild-type and IL-10 deficient mice in response to forskolin (10^{-4} M).

In IL-10 deficient mice, inhibition of AMPK with 6-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-ylpyyrazolo(1,5-a)pyrimidine (compound C; 75 µM) restored chloride secretion. Stimulation of AMPK activity with AICAR (1 mM) in wild-type mice inhibited chloride flux in response to forskolin. Values are means ± SE; n = 6–8 mice for each condition.
*p<0.01 compared with wild-type mice; $^{\#}$p<0.01 compared with IL-10 deficient mice.

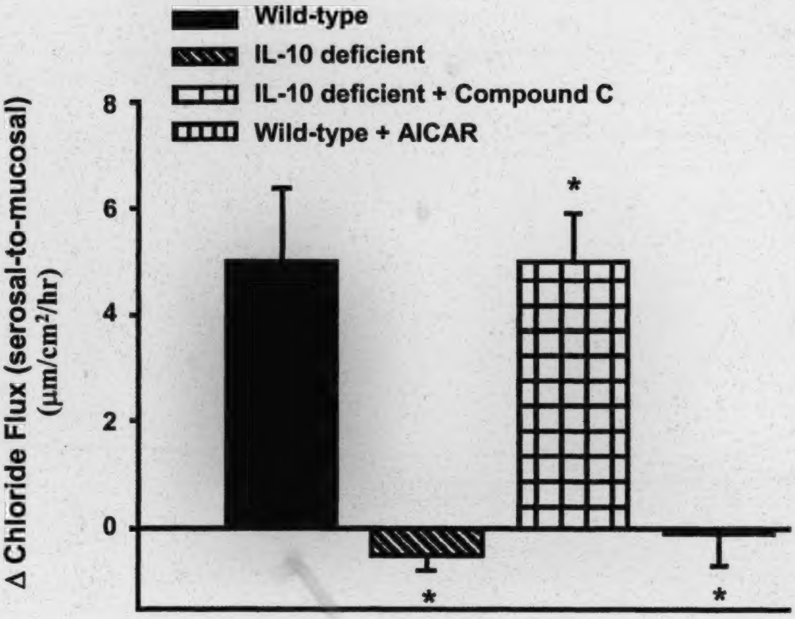


Figure 4.7. H_2O_2 and $ONOO^-$ stimulates the phosphorylation of AMPK (phospho-AMPK) in a time-dependent manner

T_{84} cells were treated with either H_2O_2 (1 mM) or $ONOO^-$ (0.1 M) for various times, and whole cell extracts were prepared for Western blot analysis. Extracts were immunoblotted with antibodies specific for the phosphorylated form of AMPK. Gels were repeated 3 times, and equal protein loading was confirmed by protein assay.

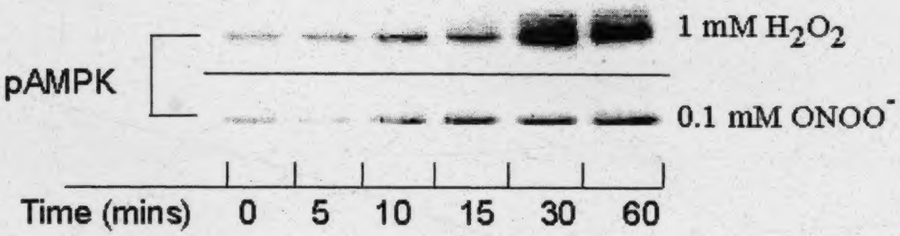


Figure 4.8. Effect of H_2O_2 on AMPK activity (A) and chloride flux (B) in T_{84} cells in the presence and absence of compound C (75 μM)

Inhibition of AMPK activity with compound C prevented the decrease in chloride flux induced by H_2O_2. Values are means ± SE; n = 5–7. *p<0.01 compared with control; #p<0.01 compared with H_2O_2.

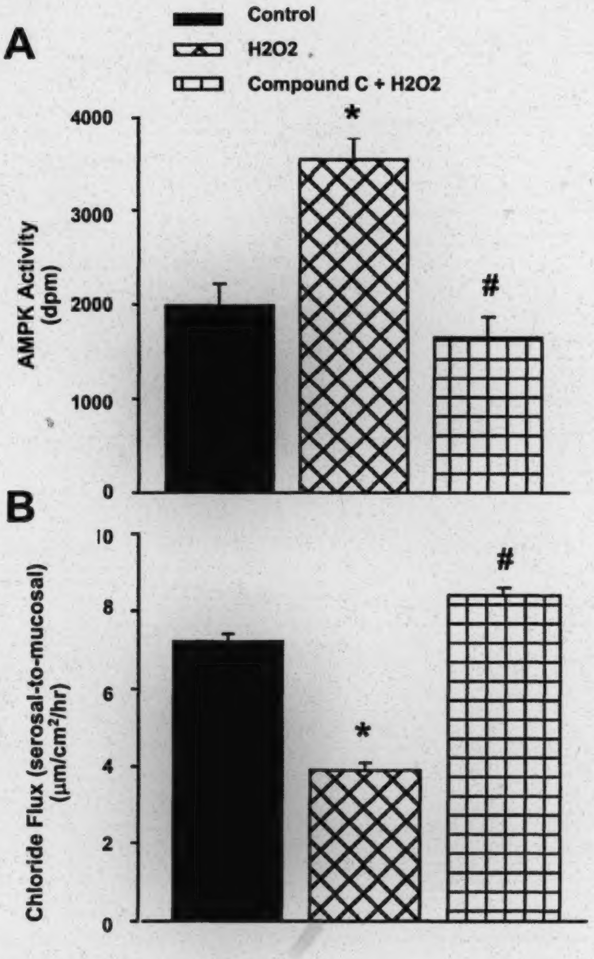


Figure 4.9. Effect of 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) treatment on apical expression of CFTR in T_{84} cells

Treatment of cells with AICAR (1 mM) for 48 hr significantly decreased the expression of CFTR. Values are means ± SE of triplicate monolayers in 3 separate experiments.
*p<0.01 compared with control monolayers.

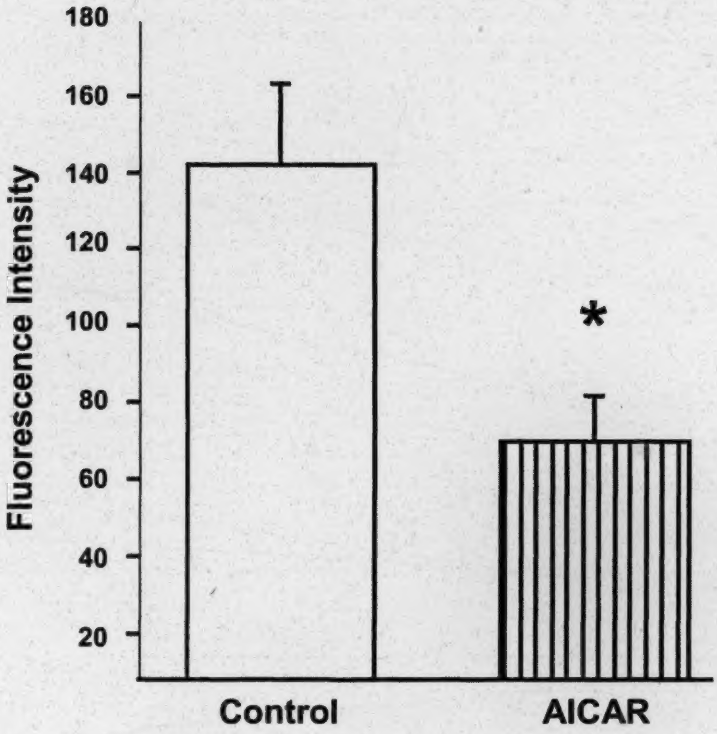


Table 4.1. Electrical parameters in the presence of indomethacin (1 µM) in proximal colon from IL-10 deficient mice

Values are means ± SE; n=4 mice. Delta was calculated as (value obtained in forskolin period) - (value in basal period). Paired t-tests were used to compare basal and forskolin periods within each group.
*p<0.01 compared with basal period.
PD, potential difference; Isc, short-circuit current; G, tissue ion conductance.

Group	**Period**	**PD (mV)**	**Isc µA/cm^2)**	**G (mS/cm^2)**
IL-10 deficient (n=4)	**Basal**	**1.1 +/- 0.1**	**15.6 +/- 1.7**	**9.8 +/- 1.7**
	Forskolin	**2.3 +/- 0.1**	**21.2 +/- 1.4**	**9.7 +/- 1.2**
	Delta	***1.2 +/- 0.1***$^{+}$	***5.6 +/- 1.8***$^{+}$	***-0.1 +/- 1.5***

Table 4.2. Electrical parameters and chloride fluxes in T_{84} monolayers

Values are means ± SE; n=5 control mice, n=8 AICAR mice. J_{sm}, serosal-to-mucosal chloride flux. Delta was calculated as (value obtained in forskolin period) - (value in basal period). Paired t-tests were used to compare basal and forskolin periods within each group.
*p<0.01 compared with basal period; $^{+}$p<0.01 compared with control.

Group	**Period**	**PD (mV)**	**Isc (μA/cm^2)**	**G (mS/cm^2)**	**J_{sm} (μEq/cm^2/hr)**
Control (n=5)	**Basal**	**2.9 +/- 0.3**	**2.1 +/- 0.2**	**0.8 +/- 0.1**	**1.3 +/- 0.4**
	Forskolin	**23.9 +/- 1.9***	**36.4 +/- 2.0***	**1.5 +/- 0.1***	**3.4 +/- 0.6***
	Delta	***20.9 +/- 1.7***	***34.2 +/- 1.9***	***0.8 +/- 0.1***	***2.1 +/- 0.7***
AICAR (n=8)	**Basal**	**0.6 +/- 0.1^{+}**	**1.0 +/- 0.1^{+}**	**2.0 +/- 0.2^{+}**	**1.5 +/- 0.3**
	Forskolin	**7.3 +/- 1.6^{*+}**	**16.8 +/- 3.3^{*+}**	**3.0 +/- 0.4**	**2.5 +/- 0.4**
	Delta	***6.7 +/- 1.5^{+}***	***15.7 +/- 3.2^{+}***	***1.0 +/- 0.4***	***1.0 +/- 0.3^{+}***

CHAPTER 5

5-aminoimidazole-4-carboxamide riboside (AICAR) enhances GLUT2-dependent jejunal glucose transport: a possible role for AMPK

Biochem J. 2005 Jan 15;385(Pt 2):485-91.

5.1. INTRODUCTION

Maintaining sufficient stores of ATP must be considered one of the fundamental requirements for cell survival. The myriad of energy-dependent processes requiring ATP include all manner of cellular operations, from gene transcription and translation to osmoregulation. Therefore, it is critical for the cell to sense and respond to alterations in energetic status. The AMP-activated protein kinase (AMPK) appears to act as a master switch able to maintain energy balance within cells by regulating rates of both ATP-consuming and ATP-generating pathways[1]. Present in all eukaryotic cells, AMPK is a serine/threonine kinase that exists as a heterotrimer composed of a catalytic α subunit and regulatory β and γ subunits[2]. Activation of AMPK involves an allosteric mechanism by which AMP binds to AMPK, in addition to phosphorylation of a threonine residue on the catalytic subunit of AMPK catalyzed by an upstream kinase, AMPK kinase (AMPKK)[3]. However, although it was initially thought that AMPK responded only under conditions of metabolic stress as a result of elevated intracellular AMP:ATP ratio[4], it is now known that AMPK responds to various stimuli that do not alter ATP levels, suggesting the involvement of other intracellular signaling pathways[5]. Recently, we characterized the role of AMPK in the intestinal epithelium as it pertained to ion secretion through the cystic fibrosis transmembrane conductance regulator (CFTR), and demonstrated that this energy-dependent ion secretory process was inhibited by AMPK[6]. However, inhibition of energy-consuming processes represents only one-half of the dual mandates of AMPK. The importance of AMPK in activating ATP-producing pathways has been demonstrated in a number of tissue and cell types including promoting fatty acid oxidation in the liver[7], and in stimulating glucose uptake by skeletal muscle[8,9].

Glucose absorption by the intestinal enterocyte involves at least two modes: transport of glucose coupled to the passage of sodium by the energy-dependent glucose transporter, SGLT1[10] [11], with an apparent K_m of 8-23 mM; and a diffusive route of entry (GLUT2), non-saturable, with a K_m between 30 and 50 mM[12]. Although the appearance of GLUT2 in the brush-border membrane

(BBM) had originally been reported in animal models of diabetes, and was presumed to be a pathological adaptation[13], subsequent studies have reported the recruitment of GLUT2 to the BBM as an adaptive measure in response to such stimuli as high luminal glucose concentrations[14], stimulation with phorbol 12-myristate 13-acetate (PMA)[15], and most recently, infusion of the enteric peptide hormone glucagon-like peptide 2 (GLP-2)[16]. Experiments performed in this study demonstrate that activation of AMPK induces glucose uptake by the intestine by increasing the recruitment of GLUT2 to the BBM, while concurrently reducing total cellular SGLT1 protein levels. We conclude from this study that AMPK promotes glucose uptake by the intestine in an energy-independent fashion while also acting to down-regulate active transport that would further stress an already metabolically compromised cell.

5.2. EXPERIMENTAL PROCEDURES

Animals

129 Sv/Ev mice were housed behind a barrier under specific pathogen-free conditions. The mice had *ad libitum* access to autoclaved 9% fat rodent blocs and sterile filtered water. The facility's sanitation was verified by Health Sciences Lab Animal Services at the University of Alberta (Edmonton, Alberta, Canada). All experiments were performed according to the institutional guidelines for the care and use of laboratory animals in research and with the permission of the local ethics committee.

Epithelial Glucose Uptake

Mice were sacrificed by cervical dislocation, and a 5 cm segment of jejunum 3 cm distal to the ligament of treitz was removed. Jejunal tissue was mounted in Lucite chambers exposing mucosal and serosal surfaces to 10 mL of oxygenated Krebs buffer (in mmol/L: 115 NaCl, 8 KCl, 1.25 $CaCl_2$, 1.2 $MgCl_2$, 2 KH_2PO_4, 25 $NaHCO_3$; pH 7.35). The buffers were maintained at 37°C by a heated water jacket and circulated by CO_2/O_2. Fructose (10 mmol/L) was added to the serosal and mucosal sides. The spontaneous transepithelial potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate short-circuit current (Isc) with an automatic voltage clamp (DVC 1000 World Precision Instruments, New Haven, CT), except for 5-10 sec every 5 min when PD was measured by removing the voltage clamp. Tissue ion conductance (G) was calculated from PD and Isc according to Ohm's Law. For the measurement of basal glucose fluxes, the tissue was clamped at zero voltage by continuously introducing an appropriate Isc with an automatic voltage clamp, except for 5-10 sec every 5 min when PD was measured by removing the voltage clamp. Tissue pairs were matched for conductance and discarded if conductance varied by > 20%. 5 μCi of the non-metabolizable glucose analogue 3-O-Methyl-d-[1-^{3}H] glucose (NEN, Boston, MA, USA) was added to the either the serosal or mucosal side after mounting and the tissue was allowed to equilibrate for 20 min. Net directional flux from mucosal-to-serosal surface was determined for conductance-matched tissues by measuring four consecutive five-min fluxes prior to the addition of AICAR (2.5 mM) and four five-min fluxes following the addition of the AMPK activator. To measure AICAR-dependent glucose transport in conjunction with p38 MAPK-inhibition, jejunum was incubated with the p38 MAPK inhibitor SB203580 (20 μM) for 30 min prior to subsequent treatment.

Glucose uptake in response to AICAR is reported as the difference between the averaged values of the four consecutive five-min fluxes prior to the addition of the drug and the averaged values of the four consecutive five-min fluxes post-treatment.

Western Blotting

Jejunal mucosa was collected by scraping with a microscope slide and suspended in 0.5 ml Mono-Q buffer. Following collection, tissue suspensions were sonicated on ice and protein concentrations determined using the Bradford method. Samples were separated by SDS-PAGE and either Coomassie-stained (to ensure even loading of lanes) or transferred onto PVDF membrane (Millipore). Membranes were blocked for 2 hr with 3% skim milk-TTBS (20 mM Tris, 0.5 M NaCl, 0.05% Tween 20, pH 7.4) and incubated overnight at 4°C with primary antibody (diluted to manufacturer's specifications). Membranes were washed 3 times with water and incubated for 2 hr with goat anti-rabbit secondary antibodies (Biorad; 1:2500 dilution), followed by 2 washes with TTBS. Autoradiography was performed on Kodak X-OMAT AR film using a chemiluminescence kit (Lumi-light, Amersham). Five antibodies were employed in this study, recognizing SGLT1 (Chemicon, Temecula, CA, USA), GLUT2 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), AMPK phosphorylated at thr172, phospho-Acetyl Co-A Carboxylase, and phospho-p38 (the latter three antibodies all having been purchased from Cell Signaling Technologies, Beverly, MA, USA), respectively. Where Western Blots are presented as representative data our findings were reproducible in at least two successive experiments.

Biotinylation of surface proteins

Proteins expressed on the apical surface of jejunal enterocytes were labeled with N-hydroxysuccinimido (NHS)-SS-biotin (Jackson Immunoresearch, West Grove, PA, USA) introduced into the intestinal lumen. At the end of the incubation the intestine was cooled on ice. The luminal solution containing NHS-SS-biotin, 1.5 mg/ml in 10 mM triethanolamine/2.5 mM $CaCl_2$/250 mM sucrose buffer (pH 9.0), was introduced into the lumen and left for 30 min. The lumen was then flushed with a PBS/100 mM glycine buffer to quench the free biotin before two final washings with PBS. Mucosal scrapings were then used to make protein extracts as described below.

Isolation of biotinylated proteins

Proteins were extracted from the homogenate for 1 hr at 4°C using the following buffer: 1.0% Triton X-100, 150 mM NaCl, 5 mM EDTA and 50 mM Tris, pH 7.5. After centrifugation at 14 000 g for 10 min the supernatant was collected and incubated overnight with streptavidin beads. After washing twice with the Triton X-100 buffer to remove non-linked protein, the beads were washed with a high-salt buffer (500 mM NaCl) and finally with a no-salt buffer (10 mM Tris, pH 7.5). The isolated biotinylated proteins were then solubilized in SDS sample buffer to be run on SDS/PAGE for Western blotting. Running samples of the supernatant after spinning down the streptavidin-coated beads on the same Western blots as samples of recovered biotinylated protein made comparisons of total cell GLUT2 with apical GLUT2.

RNA extraction and RT-PCR

Jejunal mucosa was collected in 1 mL Trizol reagent (GIBCO/BRL). RNA was isolated using a standard phenol/chloroform extraction. Briefly, mucosal scraping was vortexed extensively in Trizol before centrifugation at 14 000 g for 10 min at 4°C. Chloroform (200 μL) was added to the pellet, the mixture was spun at 12 000 g for 15 min at 4°C, and the top layer transferred to a new tube. An equal volume isopropanol was added to extract the RNA, and after centrifugation (12 000 g, 10 min, 4°C,) the pellet was washed one time with 75% ethanol.

cDNA was synthesized using the SuperScript preamplification system (GIBCO/BRL). Specific oligonucleotide primers were synthesized based on the published sequence for murine SGLT1[17]. Primer F1 was 5'-GACATCTCAGTCATCGTCATC-3' (forward) and primer F2 5'-TGTGATTGTATAAAGGGCAGTG-3' (reverse). PCR conditions were as follows: 94°C for 2 min; 21 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; and 72°C for 10 min. Amplification of a housekeeping gene, β-actin, was used as an internal PCR control. PCR products were visualized on a 2% agarose gel with 0.50 μg/mL ethidium bromide.

AMPK Activity Assay

AMPK enzyme activity was assayed as previously described[6]. Briefly, jejunal segments were excised and epithelial cells isolated using an EDTA/DTT buffer. Isolated epithelial cells were incubated at 37°C in a Krebs-HEPES buffer and treated as indicated in figure legends. Subsequent to treatment, an equivalent volume of ice-cold homogenization buffer (in mmol/L): 50 Tris HCl, 250 mannitol, 1 EDTA, 1 EGTA, 50 NaF, 5 $Na_4P_2O_7 \cdot 10\ H_2O$, 1 PMSF, 1 DTT, and 10% glycerol, 0.1% Triton X-100, and 1 μl/ml protease inhibitor cocktail (Sigma, St. Louis, MO) was added directly to the cells prior to snap-freezing in liquid nitrogen. The crude homogenate was sonicated with 4 pulses of 3 sec each before centrifugation at 18 000 g for 3 min. Protein concentrations were then determined using the Bradford method, and each sample was diluted accordingly to an equivalent protein concentration. To 200 μL of that sample was added 2 μL α-AMPK antibody (Cell Signaling Technologies, Beverly, MA, USA), and the immunoprecipitation was incubated overnight at 4°C with gentle mixing. After 12 hr immunoprecipitation, 30 μL of protein A beads (50% slurry) were added to each sample and incubated for 2 hr at 4°C with gentle mixing. The assay was begun with the addition of immunoprecipitated enzyme to assay buffer (in mM: 80 HEPES buffer, 160 NaCl, 1.6 EDTA, 200 μM SAMS peptide (Alberta

Peptide Institute, Edmonton, AB, Canada), 200 μM AMP, 200 μM ATP, 16% glycerol, 0.1% Triton X-100 and 0.5 μCi ^{32}P-γ-ATP per sample). Following addition of enzyme to the reaction tube, samples were vortexed 5 sec and incubated for 10 min at 30°C. Following incubation, the reaction mixture was vortexed and spotted on P81 Whatman filter paper (Fisher Scientific, Pittsburgh, PA, USA) briefly allowed to dry, and washed three times in 1% perchloric acid before a single wash in acetone. After sufficient time to allow the filter papers to air dry, they were immersed in a scintillant-fluor cocktail and the activity of each sample was measured in a Beckman scintillation counter. Unless otherwise listed, all reagents used in this assay were purchased from Sigma.

Statistical Analysis

Data are expressed as mean ± SE, and statistical analyses were performed using the statistical software SigmaStat (Jandel Corp, San Rafael, CA). Differences between mean values were evaluated by analysis of variance or paired *t* test where appropriate. Specific differences were tested using Student-Newman-Keuls test.

5.3. RESULTS

AICAR activates AMPK in jejunal tissue

AICAR has been employed in several studies as an activator of AMPK, but AMPK-independent AICAR responses have also been reported, albeit with less frequency. To assess the activation of AMPK by AICAR in mouse jejunal tissue, mucosal scrapings of tissue incubated with AICAR were examined in Western blots. Both the phosphorylation of AMPK at Thr172 in response to 2.5 mM AICAR, as well as the phosphorylation of the well-characterized AMPK-substrate acetyl CoA carboxylase (ACC) was assessed. Phosphorylation of Thr172 is essential for AMPK activity, and numerous studies have demonstrated that the activity of AMPK is linked with the phosphorylation status of Thr172 under all conditions thus far examined[5]. Figure 5.1.A demonstrates the time-dependent phosphorylation of both AMPK and ACC in the presence of AICAR. In both experiments, the control group was jejunal tissue treated with vehicle (0.25% DMSO) for the maximum incubation period represented in the AICAR group. Phosphorylation of both AMPK and ACC was evident by 15 minutes, and increased in intensity through 30 and 45 minutes, suggesting that AICAR activates AMPK in murine jejunal tissue.

In figure 5.1.B the effect of AICAR treatment on AMPK activity is directly demonstrated by means of a kinase assay. As measured in isolated murine jejunal epithelial cells, with 30 minutes incubation (2.5 mM) the activity of the kinase is increased greater than two-fold (2.7 ± 0.49) versus vehicle-treated cells.

Effect of AICAR on jejunal glucose transport

Serosally added AICAR (2.5 mM) resulted in a 2.2-fold increase in net stimulated jejunal 3-O-methyl glucose uptake (Figure 5.2.A). The use of the glucose transport inhibitors phloridzin and phloretin were used to discriminate between the relative contributions of the SGLT1 and GLUT2 transporters to basal and stimulated jejunal glucose absorption. In the absence of AICAR under basal conditions, both inhibitors reduced basal uptake by ≥50% suggesting that under our *in vitro* conditions, both SGLT1 and GLUT2 contributed to jejunal glucose transport. In Figure 5.2.B it can be seen that the presence of the SGLT1 inhibitor, phloridzin, had no effect on the ability of AICAR to stimulate net glucose flux. Glucose flux increased to approximately 1.7 fold that seen with phloridzin alone. In contrast, the presence of the GLUT2 inhibitor, phloretin, entirely abrogated the ability of AICAR to increase net glucose flux (Figure 5.2.C), suggesting that the up-regulation of glucose transport induced by AICAR involved GLUT2-mediated transport mechanisms. Interestingly, the presence of both AICAR and phloretin resulted in almost a complete ablation of jejunal glucose transport (Figure 5.2.C), presumably occurring as a result of phloretin-mediated inhibition of luminally localized GLUT2 concurrent with the down-regulation of SGLT1 by AICAR.

SGLT1 protein and mRNA levels in response to AICAR

To confirm that the AICAR-induced increase in net glucose flux did not involve SGLT1-dependent transport, we examined cellular mRNA levels and total protein levels of SGLT1. As seen in Figure 5.3.A, AICAR treatment of jejunal tissue results in a dramatic reduction in transporter protein levels. At the earliest time-point examined (30 minutes) total cellular SGLT1 is significantly reduced, and reduction continues as incubation time with AICAR increases. By 60 minutes the transporter is nearly undetectable in comparison with vehicle-incubated tissue. To determine if the alterations in SGLT1 protein levels involved an effect at the transcriptional level, mRNA for SGLT1 was assessed by RT-PCR. As seen in Figure 5.3.B, there was no significant effect of AICAR on SGLT1 mRNA. This lack of effect of AICAR on SGLT1 mRNA suggests an alternative regulatory mechanism may have been stimulated by AICAR that involved either alterations in SGLT1 mRNA translation or protein degradation.

Effect of AICAR on GLUT2 Localization

Together, the findings that phloridzin did not inhibit AICAR-stimulated glucose jejunal uptake combined with the observation that AICAR reduces

SGLT1 transporter levels suggested the involvement of GLUT2 in AICAR-stimulated glucose uptake. Previous studies have reported that glucose absorption in the intestine can be enhanced by the translocation of GLUT2 to the apical membrane[12,15,16]. To assess the possibility that this might be a contributing mechanism to AICAR-dependent absorption, luminal proteins were labeled with biotin, extracted, and examined by Western blotting. As Figure 5.4 shows, incubation with AICAR induces no discernable alteration in the amount of total cellular GLUT2. However, when surface-localized proteins were examined for the presence of GLUT2, we observed a significant increase in the amount of GLUT2 found in the brush border fraction in the presence of AICAR. This would suggest that the increase in net glucose flux occurred as a function of increased insertion of GLUT2 into the BBM.

p38 MAPK activation is required for AICAR-stimulated glucose transport

Previous studies have suggested that p38 MAPK activation results in increased GLUT2 levels in the BBM[15]. To determine if p38 MAPK was involved in the upregulation of glucose flux induced by AICAR in jejunal tissue, we examined phosphorylation of the kinase in response to treatment with AICAR. As seen in Figure 5.5.A, at 30 minutes AICAR treatment resulted in enhanced phosphorylation, suggesting that p38 MAPK was activated by AICAR. To investigate the role of p38 MAPK, jejunal tissue was pre-treated with 20 μM SB203580, a p38 MAPK inhibitor, and subsequently treated with AICAR. As seen in Figure 5.5.B, treatment with 20 μM SB203580 significantly attenuated the stimulation of glucose transport in response to AICAR, strongly suggesting a role for p38 MAPK in modulating GLUT2 response to AICAR. SB203580 alone did not alter jejunal glucose transport to a statistically significant degree, although a slight reduction was observed. This is in keeping with the demonstrated role of p38 MAPK in stimulating intrinsic basal transporter activity, as seen in GLUT4 regulation [35].

Effect of the p38 MAP kinase inhibitor, SB203580, on AMPK activation and translocation of GLUT2

In that previous studies have shown SB203580 to inhibit nucleoside transport, we assessed AMPK activation (by ACC phosphorylation) under the same conditions that were used for glucose transport, (30 minutes SB203580 pretreatment followed by AICAR stimulation) to ensure that the ability of SB203580 to inhibit the AICAR-induced effect was not due to a reduction in AICAR entry. As illustrated by western blot analysis in Figure 5.5.C, SB203580 did not alter phosphorylation of the AMPK substrate ACC in response to AICAR, suggesting that AICAR was still able to activate AMPK in the presence of SB203580.

To identify the role of p38 MAPK in modulating AMPK-dependent glucose uptake we examined GLUT2 translocation to the BBM in the presence of the p38 inhibitor SB203580. As seen in Figure 5.6, inhibition of p38 MAPK did not prevent AICAR-dependent GLUT2 translocation, suggesting that the role of p38 MAPK in modulating glucose transport may be a post-translocation event.

To reinforce our hypothesis that GLUT2 translocation to the BBM is attributable to AMPK activation, figure 6 also includes jejunal tissue treated with the anti-diabetic drug metformin, a known activator of AMPK. The mechanism of activation of AMPK by metformin was recently described[18] and is independent of the mechanism by which AICAR activates AMPK. Our results indicate that metformin is a potent inducer of GLUT2 translocation to the BBM, data which is in keeping with a 1994 report indicating a role for GLUT2 in luminal glucose clearance in response to treatment with metformin[19].

5.4. DISCUSSION

In the present study, we have demonstrated that activation of AMPK with AICAR in murine jejunal tissue results in an increase in net glucose flux that can be attributed to an increased amount of GLUT2 in the BBM. Glucose is actively absorbed across the intestinal epithelium by a sodium-dependent mechanism. The rate-limiting step in absorption is sodium-coupled uptake of the solute at the apical membrane which is driven by the electrochemical gradient for sodium maintained by the basolateral Na-K-ATPase pump[20]. Sodium must be extruded from the cell by Na-K-ATPase to maintain the sodium gradient; by virtue of this, all sodium-coupled solute absorption requires an input of cellular energy. Hence, under conditions of metabolic stress the cell faces a conundrum; it is imperative that cellular nutrient uptake, and therefore ATP levels, are increased, but the absorptive process *per se* is an energy-dependent process. However, in responding to an ATP deficit by altering the localization of a diffusive, high-capacity glucose transporter the cell circumvents this dilemma, in that GLUT2 facilitates glucose absorption by an energy-independent mechanism.

Data presented in this study strongly support our hypothesis that activation of AMPK results in enhanced glucose flux by a non-energy dependent mechanism involving GLUT2. This is evidenced by the observation that AICAR-stimulated glucose uptake was inhibited by phloretin, but not phloridzin, suggesting that SGLT1 was not involved. Further, the finding that levels of biotinylated GLUT2 were increased in the BBM in response to two AMPK activators also supports the hypothesis that GLUT2 responds to AMPK activation. Previous immunohistochemistry studies have shown that a substantial amount of GLUT2 protein can be seen lying just below the brush border surface in the region of the terminal web, and is rapidly inserted into the apical membrane in response to either high luminal glucose loads or the presence of GLP-2[16,21]. We extend these findings to demonstrate that GLUT2 insertion into the brush border membrane also occurs in response to AMPK activation. Interestingly, a 1994 study examining the effect of the anti-diabetic drug metformin on intestinal glucose transport demonstrated increased glucose disappearance from the jejunal lumen, as evidenced by the enhanced uptake of 2-deoxy-D-glucose. The choice of a GLUT2-selective substrate suggested the involvement of GLUT2 rather than SGLT1[19]. Not until 2001, however, was it demonstrated that AMPK activation was a primary mechanism of metformin action[22]. That AMPK acts in this fashion represents an elegant, effective response to a potential multitude of intestinal pathologies where energy metabolite levels are altered.

In addition to AICAR effects on GLUT2 localization, incubating tissue with AICAR dramatically reduced levels of cellular SGLT1, but had no effect on mRNA levels, indicating that under the conditions of our study transcriptional inhibition of SGLT1 did not occur. AMPK has been well documented as a regulator of transcriptional activity[23,24]. In their recent studies, Wang et al. proposed a novel function for AMPK as a regulator of cytoplasmic HuR levels, which in turn influenced the mRNA-stabilizing function of HuR and the stability of HuR target transcripts[25,26]. Importantly, HuR has been demonstrated to bind a stabilizing domain in the 3' untranslated region SGLT1 mRNA[27]. In light of this, both destabilization of SGLT1 mRNA and inhibition of translation may be contributing factors to SGLT1 protein down-regulation. However, given the relative abundance of SGLT1 mRNA remaining after 60 minutes of AICAR incubation, post-transcriptional events are more likely to play a significant role in the reduction in total cellular SGLT1. Recent reports in the literature indicate a possible linkage between AMPK and the mammalian target of rapamycin (mTOR) signaling pathway[28,29]. mTOR is a serine-threonine kinase that functions as a central element involved in the control of cell growth and development[30]. AICAR-mediated inhibition of mTOR-dependent translation is a possible mechanism that would explain the discrepancy between mRNA and total cellular protein levels. Further studies are required to resolve this.

While the activation of p38 MAPK has been linked with AMPK-mediated alterations in glucose transport, the exact nature of the cross-talk between these two pathways, and the role played by p38 MAPK in modulating AMPK-dependent glucose transport is less clear[31,32]. AMPK activation and p38 MAPK activity have been associated in a number of systems[33]. Further, inhibition of p38 MAPK with SB203580 has previously been shown to inhibit the effect of AICAR on glucose uptake in Clone 9 cells[34]. Our data is similar to that of Xi et al[34], in that inhibiting p38 MAPK attenuated the AICAR-stimulation of glucose flux in our system. Interestingly, studies by Helliwell and Kellett demonstrated that activating p38 MAPK in intestinal tissue resulted in enhanced GLUT2 levels and activity in the BBM[15]. They postulated that the observed increase in intestinal hexose absorption induced by physiological stress could be mediated by p38 MAPK. Our findings include a prominent role for p38 in regulating AMPK-dependent glucose transport, but it would seem that this role would exclude participation in the translocation of GLUT2 to the BBM. We demonstrated that AICAR induces GLUT2 translocation to the BBM even in the face of p38 inhibition, suggesting instead that the role of p38, as it pertains to AMPK, in regulating glucose transport is intrinsic to transporter activity. In a related study, Somwar et al. report that activation of GLUT4 (an SB203580-sensitive event,) follows GLUT4 translocation to the BBM and that both mechanisms contribute to the stimulation of glucose uptake by insulin in muscle[35]. Our findings suggest that a mechanism similar to the one proposed by Somwar could be extended to the regulation of GLUT2 in the intestine.

Evidence that p38 MAPK effects on intestinal glucose transport did not involve regulation of the AMPK-dependent translocation event suggests that p38 MAPK activation occurred either downstream of, or parallel to, AMPK activation. Interestingly, our findings also imply a more complex relationship of cross-talk between the respective kinase pathways, as inhibition of p38 MAPK, in and of itself, appeared to be sufficient to activate AMPK and furthermore, simultaneous p38 MAPK inhibition and AICAR stimulation appeared to potentiate phosphorylation of the AMPK substrate ACC.

AMPK appears to have a role in regulating whole body energy metabolism as well as serum glucose levels, in that activation of AMPK results in enhanced glucose uptake by skeletal muscle[7], increased fatty acid oxidation in skeletal muscle and liver[8,9], and inhibiting glucose production by the liver[36]. Furthermore, AMPK is involved in the regulation of insulin secretion and insulin gene expression[37], and also appears to have a critical role in regulating food intake[38]. Data from our study demonstrates that AMPK activation in the intestine also enhances net glucose uptake; thus, it is possible that AMPK activity in the intestine may have a critical role in contributing to the control of whole body energy metabolism. Although the use of 3-0-methyl glucose in our study does not allow for the examination of intestinal glucose utilization, as this is a non-metabolizable sugar, previous studies examining the effect of metformin, another activator of AMPK, have shown that metformin increases glucose utilization by the intestine, which would contribute to a reduction in serum glucose levels [39].

In conclusion, it is becoming clear that AMPK-mediated adaptive and physiological responses occur in all tissues, with the end result being maintenance of overall body energy balance. The intestine is likely an important component to this balance, with AMPK activity playing a critical role in regulating nutrient and ion transport under both normal and pathological conditions.

5.5. REFERENCES

1. Hardie, D.G., Scott, J.W., Pan, D.A. & Hudson, E.R. Management of cellular energy by the AMP-activated protein kinase system. *FEBS Lett* **546**, 113-20 (2003).
2. Carling, D., Clarke, P.R., Zammit, V.A. & Hardie, D.G. Purification and characterization of the AMP-activated protein kinase. Copurification of acetyl-CoA carboxylase kinase and 3-hydroxy-3-methylglutaryl-CoA reductase kinase activities. *Eur J Biochem* **186**, 129-36 (1989).
3. Stein, S.C., Woods, A., Jones, N.A., Davison, M.D. & Carling, D. The regulation of AMP-activated protein kinase by phosphorylation. *Biochem J* **345 Pt 3**, 437-43 (2000).
4. Kemp, B.E., Stapleton, D., Campbell, D.J., Chen, Z.P., Murthy, S., Walter, M., Gupta, A., Adams, J.J., Katsis, F., van Denderen, B., Jennings, I.G., Iseli, T., Michell, B.J. & Witters, L.A. AMP-activated protein kinase, super metabolic regulator. *Biochem Soc Trans* **31**, 162-8 (2003).
5. Carling, D. Ampk. *Curr Biol* **14**, R220 (2004).
6. Walker, J., Jijon, H.B., Churchill, T., Kulka, M. & Madsen, K.L. Activation of AMP-activated protein kinase reduces cAMP-mediated epithelial chloride secretion. *Am J Physiol Gastrointest Liver Physiol* **285**, G850-60 (2003).
7. Velasco, G., Geelen, M.J. & Guzman, M. Control of hepatic fatty acid oxidation by 5'-AMP-activated protein kinase involves a malonyl-CoA-dependent and a malonyl-CoA-independent mechanism. *Arch Biochem Biophys* **337**, 169-75 (1997).
8. Merrill, G.F., Kurth, E.J., Hardie, D.G. & Winder, W.W. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am J Physiol* **273**, E1107-12 (1997).
9. Fryer, L.G., Foufelle, F., Barnes, K., Baldwin, S.A., Woods, A. & Carling, D. Characterization of the role of the AMP-activated protein kinase in the stimulation of glucose transport in skeletal muscle cells. *Biochem J* **363**, 167-74 (2002).
10. Ikeda, T.S., Hwang, E.S., Coady, M.J., Hirayama, B.A., Hediger, M.A. & Wright, E.M. Characterization of a Na+/glucose cotransporter cloned from rabbit small intestine. *J Membr Biol* **110**, 87-95 (1989).
11. Hediger, M.A., Coady, M.J., Ikeda, T.S. & Wright, E.M. Expression cloning and cDNA sequencing of the Na+/glucose co-transporter. *Nature* **330**, 379-81 (1987).
12. Kellett, G.L. & Helliwell, P.A. The diffusive component of intestinal glucose absorption is mediated by the glucose-induced recruitment of GLUT2 to the brush-border membrane. *Biochem J* **350 Pt 1**, 155-62 (2000).
13. Corpe, C.P., Basaleh, M.M., Affleck, J., Gould, G., Jess, T.J. & Kellett, G.L. The regulation of GLUT5 and GLUT2 activity in the adaptation of intestinal brush-border fructose transport in diabetes. *Pflugers Arch* **432**, 192-201 (1996).
14. Gouyon, F., Caillaud, L., Carriere, V., Klein, C., Dalet, V., Citadelle, D., Kellett, G.L., Thorens, B., Leturque, A. & Brot-Laroche, E. Simple-sugar

meals target GLUT2 at enterocyte apical membranes to improve sugar absorption: a study in GLUT2-null mice. *J Physiol* **552**, 823-32 (2003).

15. Helliwell, P.A., Richardson, M., Affleck, J. & Kellett, G.L. Stimulation of fructose transport across the intestinal brush-border membrane by PMA is mediated by GLUT2 and dynamically regulated by protein kinase C. *Biochem J* **350 Pt 1**, 149-54 (2000).
16. Au, A., Gupta, A., Schembri, P. & Cheeseman, C.I. Rapid insertion of GLUT2 into the rat jejunal brush-border membrane promoted by glucagon-like peptide 2. *Biochem J* **367**, 247-54 (2002).
17. Tabatabai, N.M., Blumenthal, S.S., Lewand, D.L. & Petering, D.H. Mouse kidney expresses mRNA of four highly related sodium-glucose cotransporters: regulation by cadmium. *Kidney Int* **64**, 1320-30 (2003).
18. Zou, M.H., Kirkpatrick, S.S., Davis, B.J., Nelson, J.S., Wiles, I.W., Schlettner, U., Neumann, D., Brownlee, M., Freeman, M.B. & Goldman, M.H. Activation of the AMP-activated protein kinase by the anti-diabetic drug metformin in vivo: Role of mitochondrial reactive nitrogen species. *J Biol Chem* (2004).
19. Bailey, C.J., Mynett, K.J. & Page, T. Importance of the intestine as a site of metformin-stimulated glucose utilization. *Br J Pharmacol* **112**, 671-5 (1994).
20. Thomson, A.B. & Wild, G. Adaptation of intestinal nutrient transport in health and disease. Part I. *Dig Dis Sci* **42**, 453-69 (1997).
21. Affleck, J.A., Helliwell, P.A. & Kellett, G.L. Immunocytochemical detection of GLUT2 at the rat intestinal brush-border membrane. *J Histochem Cytochem* **51**, 1567-74 (2003).
22. Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M.F., Goodyear, L.J. & Moller, D.E. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* **108**, 1167-74 (2001).
23. Lee, M., Hwang, J.T., Lee, H.J., Jung, S.N., Kang, I., Chi, S.G., Kim, S.S. & Ha, J. AMP-activated protein kinase activity is critical for hypoxia-inducible factor-1 transcriptional activity and its target gene expression under hypoxic conditions in DU145 cells. *J Biol Chem* **278**, 39653-61 (2003).
24. Leff, T. AMP-activated protein kinase regulates gene expression by direct phosphorylation of nuclear proteins. *Biochem Soc Trans* **31**, 224-7 (2003).
25. Wang, W., Fan, J., Yang, X., Furer-Galban, S., Lopez de Silanes, I., von Kobbe, C., Guo, J., Georas, S.N., Foufelle, F., Hardie, D.G., Carling, D. & Gorospe, M. AMP-activated kinase regulates cytoplasmic HuR. *Mol Cell Biol* **22**, 3425-36 (2002).
26. Wang, W., Yang, X., Lopez de Silanes, I., Carling, D. & Gorospe, M. Increased AMP:ATP ratio and AMP-activated protein kinase activity during cellular senescence linked to reduced HuR function. *J Biol Chem* **278**, 27016-23 (2003).

27. Loflin, P. & Lever, J.E. HuR binds a cyclic nucleotide-dependent, stabilizing domain in the 3' untranslated region of Na(+)/glucose cotransporter (SGLT1) mRNA. *FEBS Lett* **509**, 267-71 (2001).
28. Kimura, N., Tokunaga, C., Dalal, S., Richardson, C., Yoshino, K., Hara, K., Kemp, B.E., Witters, L.A., Mimura, O. & Yonezawa, K. A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway. *Genes Cells* **8**, 65-79 (2003).
29. Inoki, K., Zhu, T. & Guan, K.L. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* **115**, 577-90 (2003).
30. Gingras, A.C., Raught, B. & Sonenberg, N. mTOR signaling to translation. *Curr Top Microbiol Immunol* **279**, 169-97 (2004).
31. Bazuine, M., Ouwens, D.M., Gomes de Mesquita, D.S. & Maassen, J.A. Arsenite stimulated glucose transport in 3T3-L1 adipocytes involves both Glut4 translocation and p38 MAPK activity. *Eur J Biochem* **270**, 3891-903 (2003).
32. Sweeney, G., Somwar, R., Ramlal, T., Volchuk, A., Ueyama, A. & Klip, A. An inhibitor of p38 mitogen-activated protein kinase prevents insulin-stimulated glucose transport but not glucose transporter translocation in 3T3-L1 adipocytes and L6 myotubes. *J Biol Chem* **274**, 10071-8 (1999).
33. Lemieux, K., Konrad, D., Klip, A. & Marette, A. The AMP-activated protein kinase activator AICAR does not induce GLUT4 translocation to transverse tubules but stimulates glucose uptake and p38 mitogen-activated protein kinases alpha and beta in skeletal muscle. *Faseb J* **17**, 1658-65 (2003).
34. Xi, X., Han, J. & Zhang, J.Z. Stimulation of glucose transport by AMP-activated protein kinase via activation of p38 mitogen-activated protein kinase. *J Biol Chem* **276**, 41029-34 (2001).
35. Somwar, R., Kim, D.Y., Sweeney, G., Huang, C., Niu, W., Lador, C., Ramlal, T. & Klip, A. GLUT4 translocation precedes the stimulation of glucose uptake by insulin in muscle cells: potential activation of GLUT4 via p38 mitogen-activated protein kinase. *Biochem J* **359**, 639-49 (2001).
36. Winder, W.W. & Hardie, D.G. AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. *Am J Physiol* **277**, E1-10 (1999).
37. Salt, I.P., Johnson, G., Ashcroft, S.J. & Hardie, D.G. AMP-activated protein kinase is activated by low glucose in cell lines derived from pancreatic beta cells, and may regulate insulin release. *Biochem J* **335 (Pt 3)**, 533-9 (1998).
38. Minokoshi, Y., Alquier, T., Furukawa, N., Kim, Y.B., Lee, A., Xue, B., Mu, J., Foufelle, F., Ferre, P., Birnbaum, M.J., Stuck, B.J. & Kahn, B.B. AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. *Nature* **428**, 569-74 (2004).
39. Bailey, C.J., Wilcock, C. & Day, C. Effect of metformin on glucose metabolism in the splanchnic bed. *Br J Pharmacol* **105**, 1009-13 (1992).

5.6. EXPERIMENTAL RESULTS

Figure 5.1.A. Time-dependent phosphorylation and activation of AMPK and phosphorylation of ACC with treatment by AICAR

Western blot of isolated jejunum incubated with 2.5 mM AICAR at 37ºC in oxygenated normal Ringer's solution for the time period indicated. In both experiments, the control group was jejunal tissue treated with vehicle (0.25% DMSO) for the maximum incubation period represented in the AICAR group. Following treatment, mucosal scrapings were analyzed by Western blot for the phosphorylation state of the indicated targets.

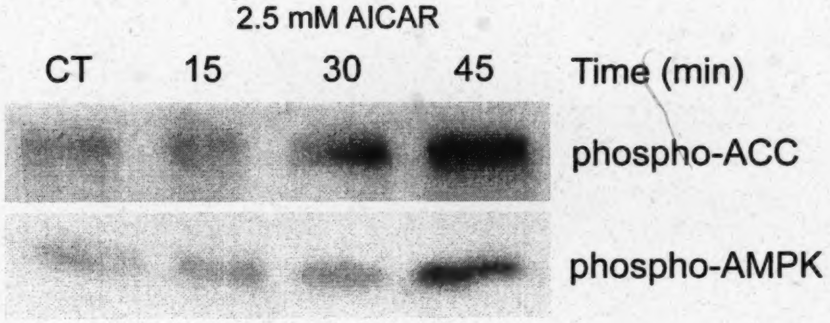


Figure 5.1.B. Time-dependent phosphorylation and activation of AMPK and phosphorylation of ACC with treatment by AICAR

AMPK activity assay of isolated jejunum incubated with 2.5 mM AICAR (30 min treatment). The control group was jejunal tissue treated with vehicle (0.25% DMSO).

Error bars represent SEM. Significance was determined by unpaired Student's t test (*p<0.05, n=5). CT: control.

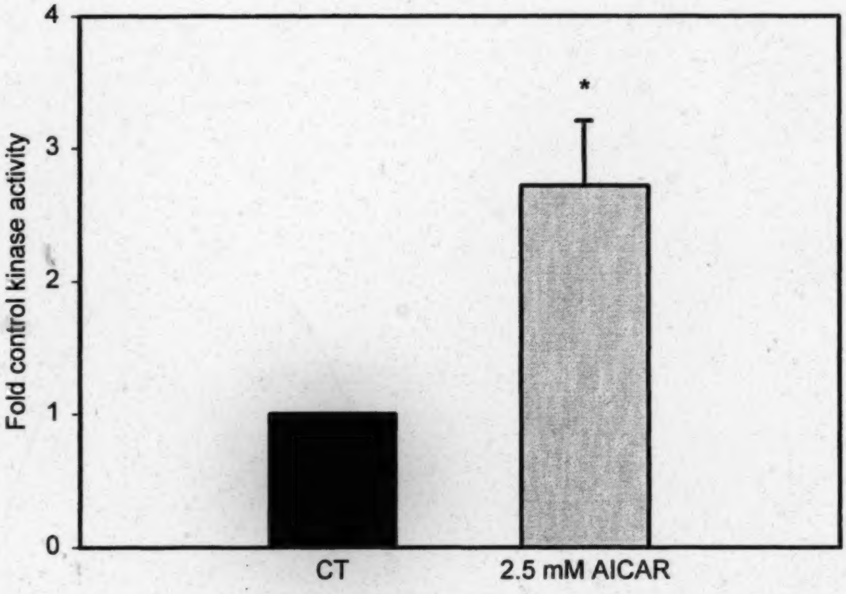


Figure 5.2.A. Uptake of 3-O-methyl glucose from isolated jejunal tissue

Jejunal tissue was mounted in Lucite chambers exposing mucosal and serosal surfaces to 10 mL of oxygenated Krebs buffer. Net directional 3-O-methyl glucose flux from mucosal-to-serosal surface was determined by measuring four consecutive five-min fluxes prior to the addition of AICAR (2.5 mM) and four five-min fluxes following the addition of the AMPK activator.

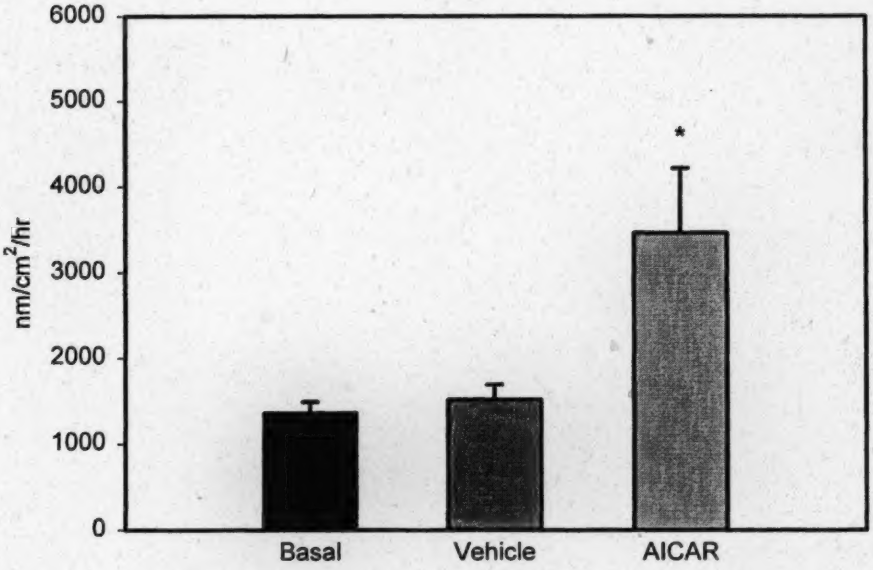


Figure 5.2.B. Uptake of 3-O-methyl glucose from isolated jejunal tissue

Values represent net directional flux as measured in 4.2.A, except in the presence of the SGLT1 inhibitor phloridzin (1 mM).

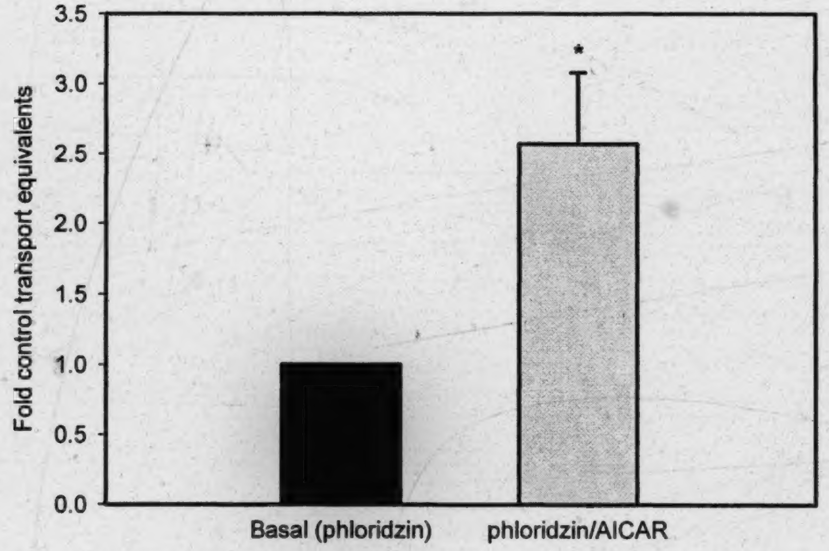


Figure 5.2.C. Uptake of 3-O-methyl glucose from isolated jejunal tissue

Values represent net directional flux as measured in 4.2.A, except in the presence of the GLUT2 inhibitor phloretin (1 mM).
Error bars represent SEM. Significance was determined by ANOVA (4.2.A.) or unpaired Student's t test (4.2.B and 4.2.C), and groups found to be statistically distinct from control are denoted by asterisk (*p<0.05, n≥5 animals per group).

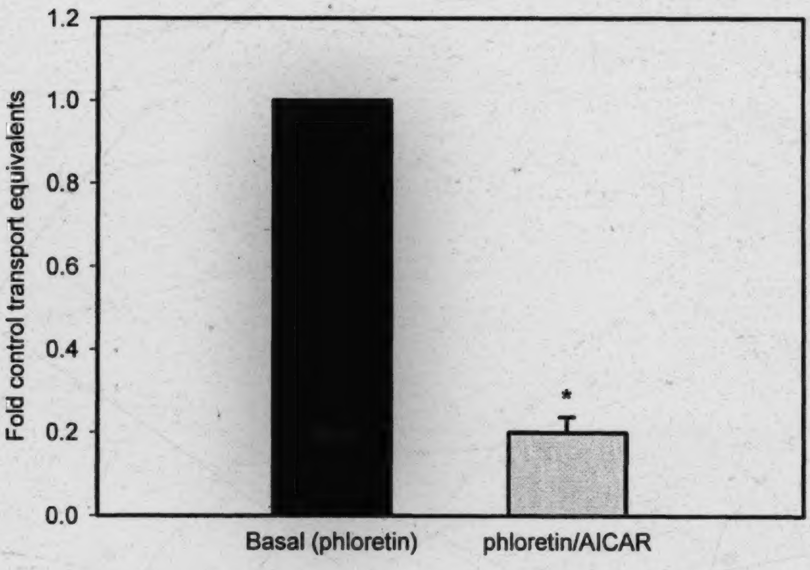


Figure 5.3.A. Alterations in SGLT1 transporter and mRNA levels in response to AICAR incubation

Western blot of isolated jejunum incubated with 2.5 mM AICAR at 37°C in oxygenated normal Ringer's solution for the time period indicated. The control group was jejunal tissue treated with vehicle (0.25% DMSO) for the respective incubation period represented in the AICAR group. Following treatment, mucosal scrapings were analyzed by Western blot for total cellular levels of SGLT1.

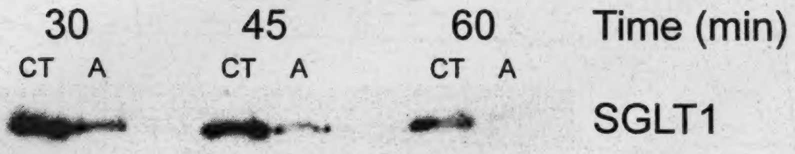


Figure 5.3.B Alterations in SGLT1 transporter and mRNA levels in response to AICAR incubation

RT-PCR experiment examining the level of SGLT1 mRNA following incubation of isolated jejunal tissue with 2.5 mM AICAR at 37°C in oxygenated normal Ringer's solution for the time periods indicated. Values are expressed as the ratio of amplified SGLT1:β-actin cDNA. Differences indicated between treatment groups were not found to be statistically different. CT: control; A: AICAR.

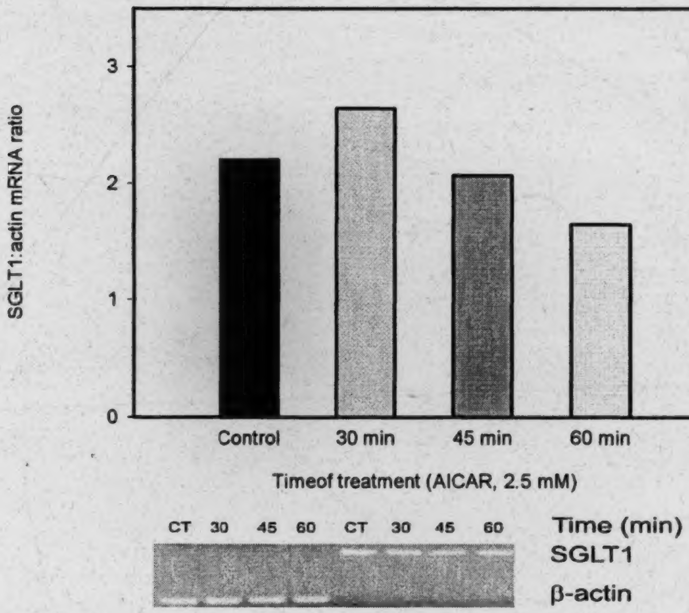


Figure 5.4. Alterations in total cellular and brush-border-localized GLUT2 in response to AICAR incubation

Western blot of isolated jejunum incubated with 2.5 mM AICAR at 37°C in oxygenated normal Ringer's solution for 45 min. Luminal proteins were labeled with biotin and examined by Western blotting for alterations in GLUT2 protein. Those lanes labeled "whole fraction" are representative of the tissue preparation prior to extraction of the biotinylated proteins, and are taken as indicative of total cellular levels of GLUT2. Those lanes labeled "surface biotinylated" are representative of isolated biotinylated proteins, and are representative of surface-expressed GLUT2. The control group was jejunal tissue treated with vehicle (0.25% DMSO) for an incubation period equivalent to the treatment groups. CT: control; A: AICAR.

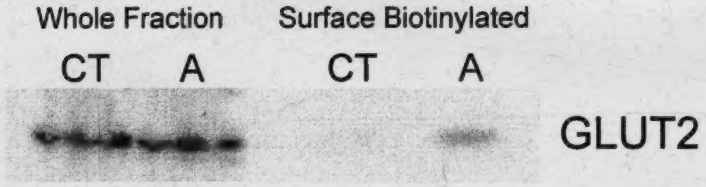


Figure 5.5.A p38 MAPK phosphorylation is required for AICAR-stimulated glucose transport

Western blot of isolated jejunum incubated with 2.5 mM AICAR at 37°C in oxygenated normal Ringer's solution for the time periods indicated. The control group was jejunal tissue treated with vehicle (0.25% DMSO) for the maximum incubation period represented in the AICAR group. Mucosal scrapings were analyzed for phospho-p38 MAPK.

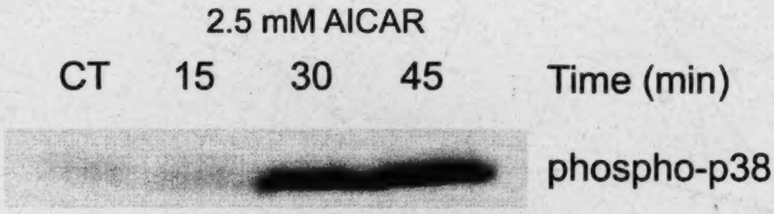


Figure 5.5.B. p38 MAPK phosphorylation is required for AICAR-stimulated glucose transport

Net directional 3-O-methyl glucose flux from mucosal-to-serosal prior to, and following the addition of AICAR (2.5 mM). Where treatment involved the p38 inhibitor SB203580 (20 μM) the jejunum was perfused *in vivo* for a period of 30 min prior to analysis of *in vitro* glucose transport as described in all other transport experiments. Error bars represent SEM. Significance was determined by ANOVA, groups found to be statistically distinct from one another share the same letter (a,b, $p<0.01$; c, $p<0.05$, $n \geq 5$ animals per group).

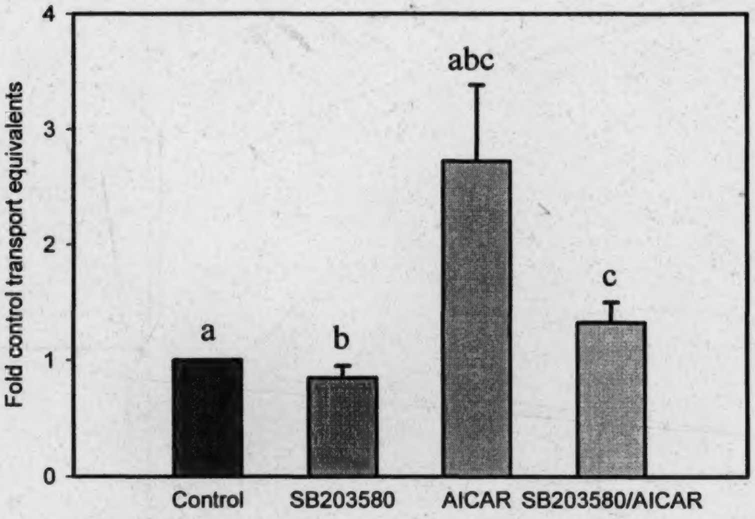


Figure 5.5.C. p38 MAPK phosphorylation is required for AICAR-stimulated glucose transport

Western blot of isolated jejunum incubated with 2.5 mM AICAR and/or SB203580 (20 μM) showing phosphorylation of the AMPK substrate Acetyl Co-A carboxylase (phospho-ACC). The control group was jejunal tissue treated with vehicle (0.25% DMSO) for a period equivalent to the treatment groups. CT: control; A: AICAR; SB: SB203580.

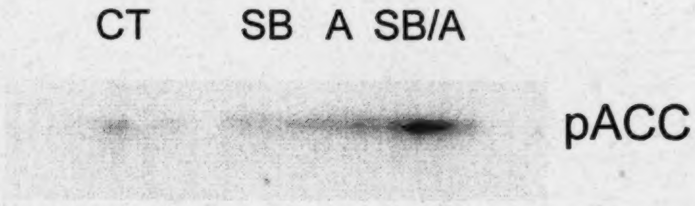
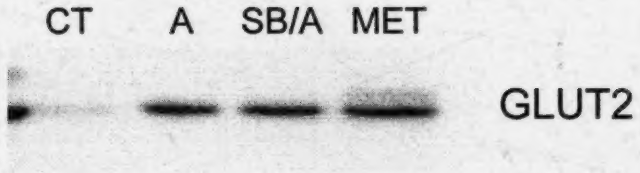


Figure 5.6. AICAR-dependent GLUT2 translocation to the apical surface is not dependent on p38 MAPK

Western blot of isolated jejunal tissue incubated with 2.5 mM AICAR (30 min) at 37°C in oxygenated normal Ringer's solution with and without 30 min pretreatment with the p38 inhibitor SB203580 (20 μM). Treatment by the AMPK activator metformin (5 mM) was performed as with AICAR. The control group was jejunal tissue treated with vehicle (0.25% DMSO) for an incubation period equivalent to the treatment groups. Lanes are representative of isolated biotinylated proteins, and are representative of surface-expressed GLUT2.

CT: control; A: AICAR; SB: SB203580; MET: metformin.



6.1. GENERAL DISCUSSION AND CONCLUSIONS

IBD is a complex, multifactorial disease the etiology of which, despite the continued best efforts of a dedicated field of researchers, remains elusive. Nonetheless, a period of intense exploration has mirrored the increase in incidence and prevalence of this disease, and this period has been fruitful; much is known about the pathophysiology of IBD and this body of information continues to grow at an astounding rate. The productivity of IBD researchers should be appreciated for two reasons; first, continued investigation will almost certainly yield a clearer, more concrete understanding of the cause of this debilitating, costly illness, and secondly, a better understanding of the pathophysiology of IBD will lend itself to an improvement in therapy for the disease. Both reasons are equally important, for in the absence of a therapy that remedies the cause of disease the best substitute is a regimen that aims to minimize the impact of that disorder on the body. Medical science has become remarkably sophisticated; technology has evolved such that the last quarter century has seen the identification of immune factors critical to the pathogenesis of disease (such as TNF[1]), as well as effective and elegant therapies that target and ablate these factors[2]. The translation of science from pathology to therapy has been astounding in its swiftness.

The research that constitutes this thesis was conducted to further the understanding of the pathogenesis of IBD; if it has helped to clarify an intricate picture by any measure it has been successful. This project was begun as an extension of an elegant study that revealed the role of the enzyme PARP in inflammation and colitis[3]. Inhibition of PARP, an important DNA repair protein, was shown to be efficacious in the treatment of IBD. And yet, there exists an understandable hesitancy to pursue PARP inhibition as a therapy in the treatment of disease; ameliorating inflammation at the expense of the maintenance of genomic integrity seems a costly exchange. Therefore, an investigation of the cellular events downstream of PARP activation became a logical extension to previous study. A great deal of the work investigating the efficacy of PARP inhibition in disease has focused on the maintenance of cellular ATP stores; PARP is a metabolically costly enzyme, and when chronically stimulated may compromise the cell in an effort to maintain genomic integrity. Thus, the question becomes: 'how does one minimize the deleterious nature of chronic PARP activation without compromising its essential function?' To that end, a large part of the research contained in this thesis was conducted to identify and characterize the role of a complex protein, AMPK, in the cascade of events between PARP activation and epithelial dysfunction; and in a greater sense, this research has also begun an investigation into the nature of AMPK activation within the context of IBD. The translation of PARP activation to AMPK activation was a natural and logical one: AMPK is exquisitely sensitive to alterations in the homeostatic ATP:AMP balance. An alteration in this equilibrium sets in motion a complex and coordinated series of events intended to restore the adenylate balance, and AMPK is the central player in this process. AMPK phosphorylates multiple targets which switch off anabolic pathways and switch on alternative catabolic pathways. Therefore, a hypothesis was generated such that with chronic intestinal inflammation PARP activity is required for AMPK activation. This hypothesis was proven correct in the work contained within this thesis, and that finding provided the cornerstone for a more detailed analysis of the nature of AMPK activation within the context of intestinal inflammation. The recognition that AMPK activity was increased with intestinal inflammation presented a vast and untapped field of investigation: fully one half of the proteins predicted from the sequencing of the human genome bear a consensus site for phosphorylation by AMPK[4]. Faced with such a vast, unpainted canvas a selection of potential substrates for regulation by AMPK were chosen to begin a characterization of the nature of AMPK activity in intestinal inflammation. Working within the paradigm of AMPK activity, two substrates were chosen for further investigation; the regulation of CFTR, an important anion secretion channel in the maintenance of epithelial function was predicted to be inhibited by AMPK – this process, while essential in the maintenance of mucosal hydration, is also dependent on ATP. Conversely, a novel AMPK target for regulation was identified in this work, GLUT2. The role of this diffusive glucose transporter in substrate absorption is beginning to emerge, and the adaptability of this mechanism to luminal and cellular glucose states[5] made it a tenable substrate for regulation by AMPK. By the findings of these studies we can say that AMPK would appear to be fulfilling its canonical role once activated in the setting of intestinal inflammation: inhibition of ATP-consuming pathways (ie. CFTR-dependent secretion) and promotion of ATP-generating pathways (ie. glucose uptake). The ramifications of these findings could be profound; an active, regulatory schema such as one conferred by AMPK could be responsible for much of what is currently viewed as epithelial dysfunction in IBD, particularly given the promiscuous nature of the kinase. To wit, preliminary studies which extend the scope of this thesis have been begun, and these studies indicate the involvement in all manner of regulation of epithelial function by AMPK, including gene transcription and the maintenance of epithelial barrier function. Furthermore, one aspect of the studies contained in chapter three of this thesis imply a mechanism of AMPK-dependent epithelial apoptosis, a potentially pathological mechanism relevant to IBD[6-8]. These findings, when taken as a whole, suggest that the activation of AMPK may be an important contributor to the pathogenesis of IBD, and certainly warrant a more detailed investigation of this pathway.

The final series of experiments contained herein were conducted to investigate the regulation of PARP by AMPK. There is a poetic continuity to the regulation of PARP by AMPK that is at once both logical and paradoxical. We describe a requirement for PARP activity in the activation of AMPK and suggest that this mechanism may be pathophysiological within the context of oxidative stress; the inference from this statement would be that AMPK activation is deleterious at the cellular level, and that targeting this process could be ameliorative where epithelial dysfunction is concerned. In fact, this may be the case, but this view is also rather limited and somewhat myopic. In investigating the regulation of PARP by AMPK the expectation should be that AMPK, if it is to play any role in the regulation of PARP, would inhibit the DNA repair enzyme. After all, the premise for investigating the regulation of AMPK by PARP rested on the realization that PARP activity resulted in serious depletion of cellular ATP stores. Our recognition of this fact made our initial investigations into AMPK-dependent PARP regulation difficult to reconcile. At least in an *in vitro* setting, AMPK appeared to potentiate the activity of PARP. Thus, what seemed to us a paradoxical feed-forward regulatory loop existed: sustained activation of the DNA repair enzyme PARP, in this context by the prolonged generation of oxidative stressors, resulted in the activation of AMPK; subsequently, the activity of AMPK resulted in an increase in PARP activity. Our *in vitro* findings were sufficiently confounding that we adopted a reductionist approach to further investigation of the relationship between AMPK and PARP, such that we could demonstrate the phosphorylation of purified PARP by AMPK, and what is more, the regulation of PARP enzymatic activity by AMPK in a dose-dependent manner. In chapter three these observations are presented in isolation; that is, the regulation of PARP by AMPK is investigated and described without consideration of the inverse relationship between the two proteins. Similarly, chapter two describes the activation of AMPK downstream of PARP activation, but similarly ignores the inverse relationship. This was done for two reasons, the first being that in isolation each regulatory aspect of what is truthfully, for lack of a better term, a bidirectional relationship has potentially significant clinical relevance. Excessive activation of AMPK due to PARP activity will certainly result in what could be interpreted as epithelial dysfunction, for example the inhibition of CFTR functions in the bowel. Conversely, should AMPK activity be impaired, the resultant impairment of PARP function could reasonably be expected to manifest pathophysiologically, perhaps through an increase in neoplastic growth by a defect in DNA repair. In fact, Peutz-Jeghers syndrome, a defect described at length in chapter three, could be the clinical manifestation of our observations to this end. The second reason for examining the two relationships apart from one another is simpler – the evolution of this project was an organic process, and as it progressed in concert with the field of research and the maturation of the author(s) what had initially seemed a straightforward and linear project became significantly more complex. To be sure, the true regulatory nature of the relationship between AMPK and PARP is not resolved by this work, and what we are left with, now, is a measure of supposition as to what the actual dynamics might be. In light of that realization, is tempting to speculate that the role of AMPK in the rescue of the cell from ATP depletion might be greater than the sum of its parts. Evidence suggests that AMPK already occupies a position as a metabolic checkpoint of sorts, coordinating sufficient energy stores with the progression through the cell cycle[9]; it may be that, in fact, AMPK coordinates the choice between cellular life and death, so to speak. When placed in context with one another, the findings in this thesis suggest that the activation of AMPK, acutely, results in a cascade of events intended to rescue the cell; chronic activation of AMPK, however, may deliver an entirely different signal to the cell, resulting in apoptosis, which is a fate preferable to the inevitable necrosis that would accompany complete wasting of ATP stores. There is evidence to support this concept, as sustained activation of AMPK has been linked with apoptosis[10,11]. Furthermore, in certain settings PARP activation seems to be a requirement for apoptosis[12,13] (although a cautious interpretation of this point is required, as others have shown PARP cleavage (and inactivation) to be necessary for apoptosis[14]).

The work conducted during these studies was intended to place AMPK within the context of IBD, and to gain a better understanding of the implications of AMPK activation under these conditions. With further study, the true nature of this process both in health and disease will be better understood, and with that better understanding will come an improvement in therapy for a complex illness.

6.2. REFERENCES

1. Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N. & Williamson, B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci U S A* **72**, 3666-70 (1975).
2. van Dullemen, H.M., van Deventer, S.J., Hommes, D.W., Bijl, H.A., Jansen, J., Tytgat, G.N. & Woody, J. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). *Gastroenterology* **109**, 129-35 (1995).
3. Jijon, H.B., Churchill, T., Malfair, D., Wessler, A., Jewell, L.D., Parsons, H.G. & Madsen, K.L. Inhibition of poly(ADP-ribose) polymerase attenuates inflammation in a model of chronic colitis. *Am J Physiol Gastrointest Liver Physiol* **279**, G641-51 (2000).
4. Hardie, D.G. Roles of the AMP-activated/SNF1 protein kinase family in the response to cellular stress. *Biochem Soc Symp* **64**, 13-27 (1999).
5. Kellett, G.L. & Brot-Laroche, E. Apical GLUT2: a major pathway of intestinal sugar absorption. *Diabetes* **54**, 3056-62 (2005).
6. Bojarski, C., Bendfeldt, K., Gitter, A.H., Mankertz, J., Fromm, M., Wagner, S., Riecken, E.O. & Schulzke, J.D. Apoptosis and intestinal barrier function. *Ann N Y Acad Sci* **915**, 270-4 (2000).
7. Gitter, A.H., Bendfeldt, K., Schulzke, J.D. & Fromm, M. Leaks in the epithelial barrier caused by spontaneous and TNF-alpha-induced single-cell apoptosis. *Faseb J* **14**, 1749-53 (2000).
8. Abreu, M.T., Palladino, A.A., Arnold, E.T., Kwon, R.S. & McRoberts, J.A. Modulation of barrier function during Fas-mediated apoptosis in human intestinal epithelial cells. *Gastroenterology* **119**, 1524-36 (2000).
9. Jones, R.G., Plas, D.R., Kubek, S., Buzzai, M., Mu, J., Xu, Y., Birnbaum, M.J. & Thompson, C.B. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol Cell* **18**, 283-93 (2005).
10. Kefas, B.A., Heimberg, H., Vaulont, S., Meisse, D., Hue, L., Pipeleers, D. & Van de Casteele, M. AICA-riboside induces apoptosis of pancreatic beta cells through stimulation of AMP-activated protein kinase. *Diabetologia* **46**, 250-4 (2003).
11. Meisse, D., Van de Casteele, M., Beauloye, C., Hainault, I., Kefas, B.A., Rider, M.H., Foufelle, F. & Hue, L. Sustained activation of AMP-activated protein kinase induces c-Jun N-terminal kinase activation and apoptosis in liver cells. *FEBS Lett* **526**, 38-42 (2002).
12. Smulson, M.E., Simbulan-Rosenthal, C.M., Boulares, A.H., Yakovlev, A., Stoica, B., Iyer, S., Luo, R., Haddad, B., Wang, Z.Q., Pang, T., Jung, M., Dritschilo, A. & Rosenthal, D.S. Roles of poly(ADP-ribosyl)ation and PARP in apoptosis, DNA repair, genomic stability and functions of p53 and E2F-1. *Adv Enzyme Regul* **40**, 183-215 (2000).
13. Simbulan-Rosenthal, C.M., Rosenthal, D.S., Iyer, S., Boulares, H. & Smulson, M.E. Involvement of PARP and poly(ADP-ribosyl)ation in the

early stages of apoptosis and DNA replication. *Mol Cell Biochem* **193**, 137-48 (1999).

14. Los, M., Mozoluk, M., Ferrari, D., Stepczynska, A., Stroh, C., Renz, A., Herceg, Z., Wang, Z.Q. & Schulze-Osthoff, K. Activation and caspase-mediated inhibition of PARP: a molecular switch between fibroblast necrosis and apoptosis in death receptor signaling. *Mol Biol Cell* **13**, 978-88 (2002).